Investigating the Porphyrias Through Analysis of Biochemical Pathways

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By Evonne Teresa Nicole Ruegg

University of Canterbury

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I would like to dedicate this thesis to my parents for their ongoing inspiration and support and for always believing in me.

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ABSTRACT

The porphyrias are a diverse group of metabolic disorders arising from diminished activity of enzymes in the heme biosynthetic pathway. They can present with acute neurovisceral symptoms, cutaneous symptoms, or both. The complexity of these disorders is demonstrated by the fact that some acute porphyria patients with the underlying genetic defect(s) are latent and asymptomatic while others present with severe symptoms. This indicates that there is at least one other risk factor required in addition to the genetic defect for symptom manifestation. A systematic review of the heme biosynthetic pathway highlighted the involvement of a number of micronutrient cofactors. An exhaustive review of the medical literature uncovered numerous reports of micronutrient deficiencies in the porphyrias as well as successful case reports of treatments with micronutrients. Many micronutrient deficiencies present with symptoms similar to those in porphyria, in particular vitamin B6. It is hypothesized that a vitamin B6 deficiency and related micronutrient deficiencies may play a major role in the pathogenesis of the acute porphyrias. In order to further investigate the porphyrias, a computational model of the heme biosynthetic pathway was developed based on kinetic parameters derived from a careful analysis of the literature. This model demonstrated aspects of normal heme biosynthesis and illustrated some of the disordered biochemistry of acute intermittent porphyria (AIP). The testing of this model highlighted the modifications necessary to develop a more comprehensive model with the potential to investigated hypotheses of the disordered biochemistry of the porphyrias as well as the discovery of new methods of treatment and symptom control. It is concluded that vitamin B6 deficiency might be the risk factor necessary in conjunction with the genetic defect to trigger porphyria symptoms.

ABBREVIATIONS

A list of abbreviations used:

AIP	acute intermittent porphyria	NAD^+	nicotinamide adenine dinucleotide
ALA	aminolevulinate	NADP(H)	nicotinamide adenine dinucleotide phosphate
ALAD-P	aminolevulinate dehydratase deficiency porphyria	ODE	ordinary differential equation
ALAS	aminolevulinate synthase	PBG	porphobilinogen
BBB	blood brain barrier	PBGD	porphobilinogen deaminase
BRENDA	braunschweig enzyme database	PCT	porphyria cutanea tarda
CEP	congenital erythropoietic porphyria	PGC-1	proliferator-activated receptor coactivator 1
CNS	central nervous system	PLP	pyridoxal 5'phosphate
CoA	coenzyme A	PPOX	protoporphyrinogen oxidase
COPASI	complex pathway simulator	TCA	tricarboxylic
CPOX	coproporphyrinogen oxidase	THFA	tetrahydrofolic acid
EPP	erythropoietic protoporphyria	ThPP	thiamine pyrophosphate
FAD	flaven adenine dinucleotide	IU	international unit
GABA	γ-aminobutyric acid	UROD	uroporphyrinogen decarboxylase
HCP	hereditary coproporphyria	UROS	uroporphyrinogen synthase
HEP	hepatoerythropoietic porphyria	UV	ultraviolet
HIV	human immunodeficiency virus	VP	variegate porphyria
HMB	hydroxymethylbilane	XLPP	X-linked dominant protoporphyria
IV	intravenous	XLSA	X-linked sideroblastic anemia

Note for ease of reading, there is a fold-out schematic representation of the heme biosynthetic pathway along with a list of abbreviations of the heme biosynthetic pathway enzymes and the corresponding porphyrias.
All other abbreviations can be accessed easily through the fold-out page at the end of the thesis.

CHAPTER 1. INTRODUCTION

1.1 Introduction

1.1.1 Porphyrins

"The porphyrin pathway is the source of the "rings of life" and its significance is interwoven in the fabric of the biological world." - Dayan and Dayan.[1]

Porphyrins are chemical compounds made up of four pyrrole rings (Figure 1.1) that are capable of binding metal ions and play essential roles in a variety of important biological reactions. They comprise a large class of deeply coloured, fluorescent red or purple crystalline pigments.[2] Their name is derived from the Greek word for purple – *porphura*. They were given this name when brilliantly coloured dyes extracted from indigo plants were found to contain porphyrins. The colourful purple hues provided by the porphyrin dyes were colourfast due to the stability of the porphyrins and were considered a luxury as a result, hence the term 'royal purple'. They have also been termed "the colours of life"[2] partly because they are responsible for the red colour of blood (hemoglobin) as well as the green colour of plants (chlorophyll). Porphyrins in addition to melanin pigments also account for the rich reds, browns and greens of the feathers of some birds.

Due to the ability of porphyrins to bind metal ions, they are capable of playing important roles in a variety of biological processes including O_2 transport (hemoglobin, myoglobin), photosynthesis (chlorophyll), electron transfers (cytochromes) as well as drug metabolism (cytochromes P450). In humans, it is the porphyrin 'heme' that is utilised in myriad crucial metabolic pathways ranging from

oxygen transport to gene regulation.[3] Heme biosynthesis is a complex eight-step pathway involving a series of porphyrin and heme precursors. The heme biosynthetic pathway is tightly regulated by its end product, heme, through feedback inhibition (Figure 2.1); this ensures these porphryin precursors do not accumulate in excess under normal conditions.

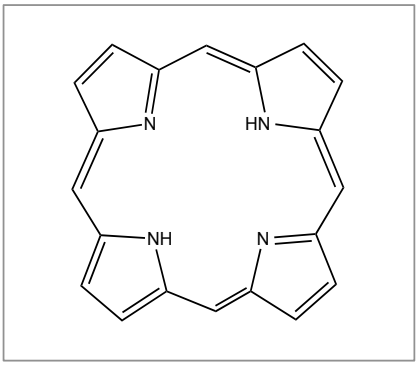

Figure 1.1 Structure of a Simple Porphyrin.

1.1.2 When Heme Synthesis Goes Wrong

Other than their role in heme synthesis, the porphyrin heme precursors have no known role in human metabolism and never accumulate under normal conditions as the heme pathway is tightly regulated. Each of the eight steps of heme biosynthesis is catalysed by a specific enzyme (Figure 2.1). Genetic mutations that result in diminished activity of one or more of these enzymes lead to a group of metabolic disorders called the porphyrias. The reduced enzyme activity can cause a 'bottleneck' in the normal flux of the heme biosynthetic pathway resulting in a buildup of porphyrins and heme precursors prior to the enzymatic 'block' in the pathway. Porphyrin and heme precursor accumulation is associated with toxic effects resulting in a wide spectrum of symptoms. Hence the term 'porphyria'.

The porphyrias can present with myriad detrimental symptoms ranging from acute symptoms, e.g. severe abdominal pain, peripheral neuropathies, seizures, hallucinations, and psychosis, to chronic symptoms presenting with extreme skin photosensitivity and resulting skin damage. The porphyrias are classified as either 'acute' or 'cutaneous' depending upon the predominant symptoms. The underlying mutation of a heme biosynthetic enzyme(s) results in predisposition for these disorders but external factors are required for the manifestation of symptoms. These external 'triggers' include drugs, chemicals, hormones, stress, fasting, and exercise. In addition, many porphyria subjects with an underlying enzyme deficiency remain asymptomatic and may never experience symptoms; because of this the exact biochemical mechanism required to initiate many of these symptoms is unknown. Further research into the biochemistry of porphyria is required to expand current knowledge of the biochemical pathogenesis of these disorders which is still lacking in many areas.

1.1.3 Porphyria – a Brief Historical Context

Porphyria is possibly most famous for its suggested role in the madness of King George III of Great Britain. Medical detectives have hypothesised that he may have had variegate porphyria as he suffered from symptoms similar to those of this acute porphyria. King George is reported to have had dark reddish urine characteristic of porphyria as well as psychiatric disturbances that are often seen in acute forms of porphyria. Although it has been suggested otherwise,[4] evidence strongly points that King George may have suffered from disturbed heme metabolism in the form of variegate porphyria. If this is the case, porphyria may have played a role in world events at the time.

The artist, Vincent van Gogh, has also been suggested to have suffered from altered heme metabolism. He suffered from episodes of acute mental derangement and disability yet was in apparent good health between episodes. The wide-spread popularity of his paintings has aroused interest in his medical problems and the role it played in his life as well as his artwork. It has been suggested in a review of his symptoms and history that acute intermittent porphyria best describes his medical crises.[5, 6] Other suggested theories to explain his symptoms include lead poisoning and excessive absinthe consumption, both of which are triggering factors of acute porphyria.

In addition to these historical examples, porphyria may currently be of more contemporary significance than previously appreciated. It has been suggested that 20% of mankind may have a predisposition to porphyria and many chronic illnesses may actually be porphyria in disguise or due to altered heme metabolism.[7]

1.1.4 Micronutrients – Treatment Potential?

The porphyrias are a group of metabolic disorders which present with a wide array of symptoms the mechanisms of which are not fully understood. The search for safe prophylaxis and treatments for these symptoms is an ongoing area of research. There have been reports that suggest the underlying mutation of the heme enzyme(s) in these disorders may be augmented by micronutrient deficiencies.[8-22] There have been reports of successful treatment of the porphyrias with high-dose micronutrient supplementation,[8, 10, 12, 13, 15, 23-55] although this is not widely acknowledged.

A better understanding of the underlying mechanisms of the porphyrias as well as current treatments is essential in understanding the pathogenesis of porphyria symptoms. Investigations into the heme pathway as well as other connected pathways that share common substrates and products will further current knowledge of the porphyrias as well as the discovery of new treatment regimes.

1.2 Aims and Objectives

The aim of this thesis is to develop a greater understanding of the porphyrias and underlying nutritional issues through:

1. Reviewing literature on the effects of micronutrients in the porphyrias with the aim of developing a testable hypothesis that could aid future treatments.

2. Developing a methodology suitable for testing the hypothesis.

CHAPTER 2. THE HEME BIOSYNTHETIC PATHWAY

2.1 Introduction

The heme biosynthetic pathway is one of the most important pathways of human metabolism. Heme is essential for biologic oxidations and is a prosthetic group in a wide variety of vital proteins including hemoglobin, myoglobin, cytochromes, catalases, and peroxidases.[56] Hemoproteins are responsible for oxidative reactions, electron transfer processes and the delivery of molecular oxygen to cells.[57] Due to the involvement of heme in myriad important cellular processes, altered heme synthesis can result in a variety of detrimental effects. The heme biosynthetic pathway is an eight-step pathway involving porphyrin and porphyrinogen precursors that is tightly regulated by the end product heme (Figure 2.1). Each step of the pathway is catalysed by a specific enzyme. Genetic mutations resulting in the diminished activity of one or more of the enzymes of this pathway are the underlying factors of the group of hereditary metabolic disorders known as the porphyrias (see Chapter 3). The deficient enzyme(s) cause a 'bottleneck' in the pathway due to up-regulation of the enzymes prior to the 'block' in an attempt to meet heme demands. This results in elevated levels of heme precursors in the steps prior to the enzyme 'block'. Each porphyria results in a unique precursor overproduction pattern depending on where the bottleneck in the pathway occurs. The porphyrias will be discussed further in Chapter 3.

The heme biosynthetic pathway is a complex pathway. A variety of cofactors are required for optimal activity of the eight enzymes involved, as well as for formation of the initial substrates glycine and succinyl-CoA.[58] These cofactor micronutrients include vitamin B6, riboflavin, biotin, pantothenic acid, thiamine, and lipoid acid, as

well as the minerals zinc, iron, and copper (Table 2.1).[58] In addition, many heme proteins that are reliant on this pathway are also required for the activation and/or utilization of some micronutrients e.g. vitamin D.[59] Due to the heme biosynthetic pathway's dependence on so many micronutrients it is possible that disordered heme metabolism may affect the balance of these essential cofactors. Could this also play a role in the manifestations of the porphyrias? A thorough understanding of the heme biosynthetic pathway will allow a better understanding of the porphyrias. This chapter will provide a background of heme synthesis with an emphasis on the cofactors involved.

2.2 Heme Biosynthesis

2.2.1 Overview

Heme biosynthesis begins with the formation of aminolevulinate (ALA), the universal precursor for all tetrapyrroles. There are two distinct pathways of ALA synthesis. In plants, algae, and most bacteria, ALA is produced from glutamate in a three-step reaction.[60] In eukaryotes, fungi, and an α-subclass of purple bacteria, ALA is produced from succinyl coenzyme-A (succinyl-CoA) and glycine.[61] This review will focus only on the latter pathway, the Shemin pathway, as it is the route that is medically significant for humans and the porphyria discussions in future chapters. In humans, succinyl-CoA is supplied from the tricarboxylic acid (TCA) cycle and glycine is found in the mitochondrial amino acid pool. The pathway begins in the mitochondria with the condensation of succinyl-CoA with glycine to produce ALA. ALA is then transported to the cytoplasm where two molecules of ALA are condensed to form porphobilinogen (PBG). Four molecules of PBG are then converted into uroporphyrinogen followed by coproporphyrinogen which is

transported back into the mitochondria where decarboxylation and oxidation steps result in the conversion of coproporphyrinogen into protoporphyrinogen and then finally protoporphyrin (Figure 2.1). The final step involves the insertion of iron

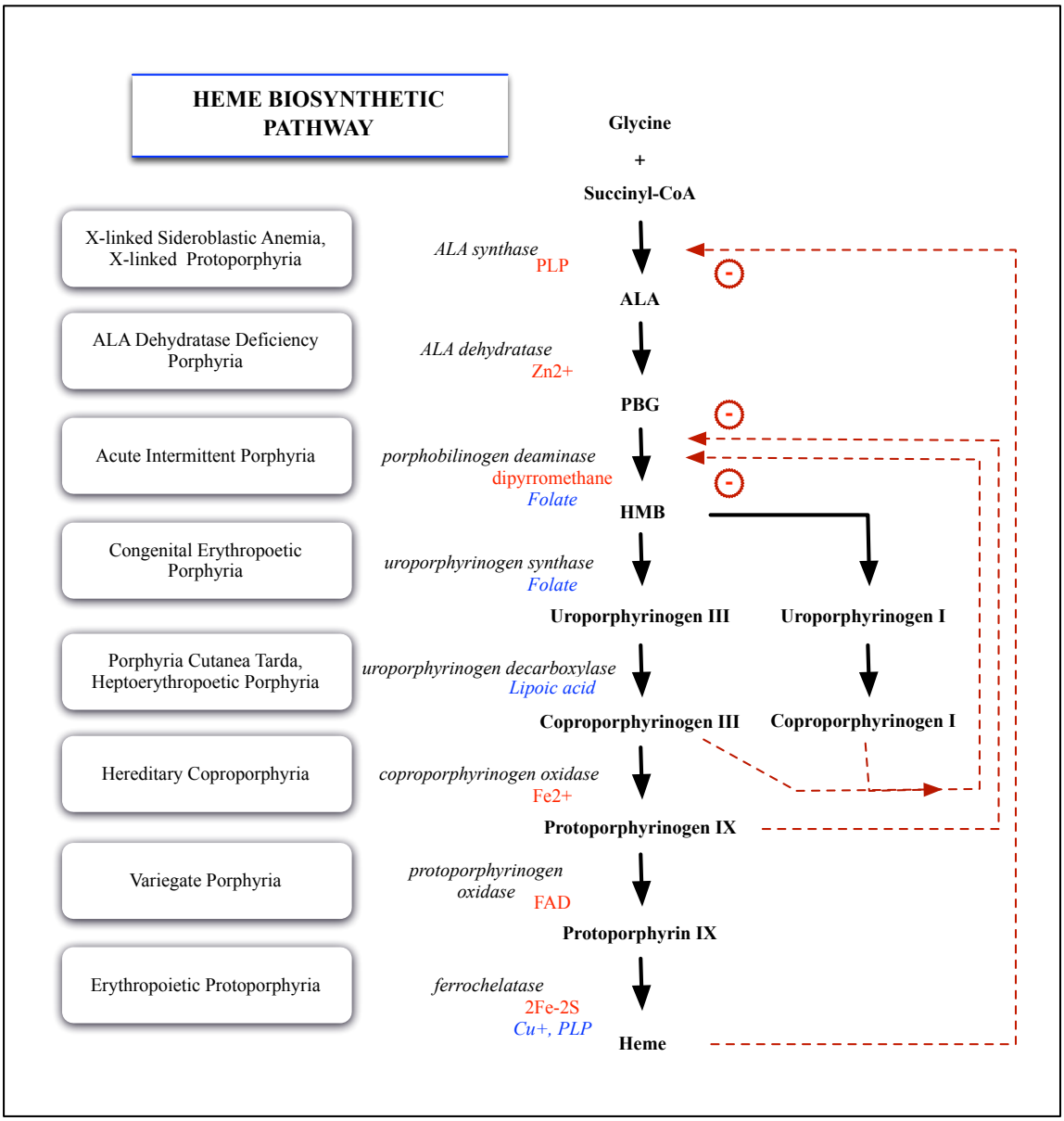

Figure 2.1 Heme Biosynthetic Pathway.

This figure illustrates the metabolic pathway of heme synthesis with the disorders relating to heme enzyme mutations reported to the left of the respective enzyme. Known cofactors are listed below the enzyme in red text and possible cofactors/activators are shown in blue text. The red-dashed arrows indicate feedback inhibition. PLP, pyridoxal 5'phosphate (vitamin B6); FAD, flavin adenine dinucleotide (riboflavin); 2Fe-2S, iron sulfur clustur.

into protoporphyrin IX to form heme. Heme can then be utilised into hemoproteins or degraded into bilirubin which is excreted as bile pigment. Porphyrinogens are important to cellular processes due to their metal-binding capability; heme is an iron-containing compound which is usually found bound to proteins involved in biological oxidations and oxygen transport. Chlorophylls are the plant version of heme, and are magnesium-porphyrin complexes. Heme provides blood with its red colour while chlorophyll is responsible for the green colour of plants. The porphyrinogen precursors, uroporphyrinogen and coproporphyrinogen, along with their isomers and intermediates, are rapidly oxidised to porphyrins when exposed to air with a net loss of six protons. These porphyrins absorb light at 400-410 nm wavelength and emit a fluorescent red colour upon exposure to these wavelengths of ultraviolet (UV) light. Porphyrins are continuously excreted from the body either through the urine or as bile pigment and have no known biological function other than as precursors for heme.[58] It is the porphyrins, rather than the porphyrinogens that accumulate in the porphyrias (Chapter 3).

Eight molecules of succinyl-CoA from the TCA cycle[62, 63] and eight molecules of glycine from the mitochondrial pool of amino acids are required to produce one molecule of heme. Therefore the biosynthesis of these two substrates is also significant to heme synthesis as the stoichiometry of these substrates has the potential to make them rate limiting.

2.2.2 Succinyl-CoA - the TCA Cycle

Succinyl-CoA is one of the initial building blocks for heme biosynthesis and is derived from the TCA cycle (Figure 2.2). Succinyl-CoA provides the main energy

source for the heme biosynthetic pathway.[64] Eight molecules of succinyl-CoA are required to form one molecule of heme, however, not all heme precursors complete the pathway to form heme as some are excreted from the body, making the total succinyl-CoA requirement per heme molecule greater than eight.[58] Succinyl-CoA is not only used in heme synthesis but is also required in ATP production and in the recycling of TCA cycle intermediates.[65] Depletion of succinyl-CoA due to increased enzymatic activity in the early steps of the heme biosynthetic pathway in the porphyrias could thus reduce succinyl-CoA availability for other reactions. The co-factor requirements of the TCA cycle are therefore relevant to heme biosynthesis due to the latter pathway's dependence on succinyl-CoA.

Acetyl-CoA is required in the first step of the TCA cycle and is provided through the transformation of pyruvate from the glycolysis pathway. This reaction is catalyzed by the pyruvate dehydrogenase enzyme complex and requires the nutrient cofactors thiamine pyrophosphate (ThPP), CoA, lipoic acid, flavin adenine dinucleotide (FAD), and nicotinamide adenine dinucleotide (NAD^+). The TCA cycle then begins with the formation of citrate from oxaloacetate and acetyl-CoA which is then transformed to isocitrate via a two-step process requiring an iron-sulfur cluster cofactor. Isocitrate is subsequently oxidatively decarboxylated to α-ketoglutarate via isocitrate dehydrogenase which requires NAD^+ as a cofactor. The conversion of α-ketoglutarate to succinyl-CoA is also an oxidative decarboxylation catalysed by the α-ketoglutarate dehydrogenase complex. This enzyme complex is similar to the pyruvate dehydrogenase complex in that it also requires ThPP, CoA, lipoic acid, FAD and NAD^+ as cofactors. Succinyl-CoA can then enter heme biosynthesis and other pathways or it can be converted back into oxaloacetate to complete the TCA cycle.

This involves a further four steps beginning with the conversion of succinyl-CoA to succinate coupled with ATP release. Succinate is then transformed to fumarate via succinate dehydrogenase, an enzyme which is also part of the electron transport chain and which requires an iron-sulfur cluster as well as FAD. Fumarate then proceeds to L-Malate and finally, via malate dehydrogenase which also utilizes an FAD cofactor as well as NAD^+, back to oxaloacetate and the beginning of the TCA cycle. Oxaloacetate is also capable of being formed from pyruvate via carboxylase in a biotin-dependent reaction. In summary, the following nutrient cofactors are involved in the TCA cycle and thus succinyl-CoA synthesis: biotin, thiamine (ThPP), pantothenate (CoA), lipoic acid, niacin (NAD^+), riboflavin (FAD) and iron-sulfur clusters (Figure 2.2).

The TCA cycle is an amphibolic pathway as its intermediates support both anabolic and catabolic processes.[66] Oxaloacetate, α-ketoglutarate, citrate, and succinyl-CoA, TCA cycle intermediates, are important precursors for the biosynthesis of porphyrins and heme, amino acids, and fatty acids (Figure 2.2).[58] Other than the porphyrins, the metabolites of these pathways turn over to provide the TCA cycle with its intermediates. Atamna et al.[58] provides a convincing argument that porphyrin/heme biosynthesis is energy consuming, that the heme biosynthetic pathway puts a high demand on the TCA cycle, and that when heme requirements are high the TCA cycle may be drained of its intermediates as well as micronutrient cofactors. Atamna et al.[58] also suggest that this might contribute to ageing. Could the disordered heme biosynthetic pathway of the porphyrias also drain the TCA cycle of its intermediates and result in cofactor depletion?

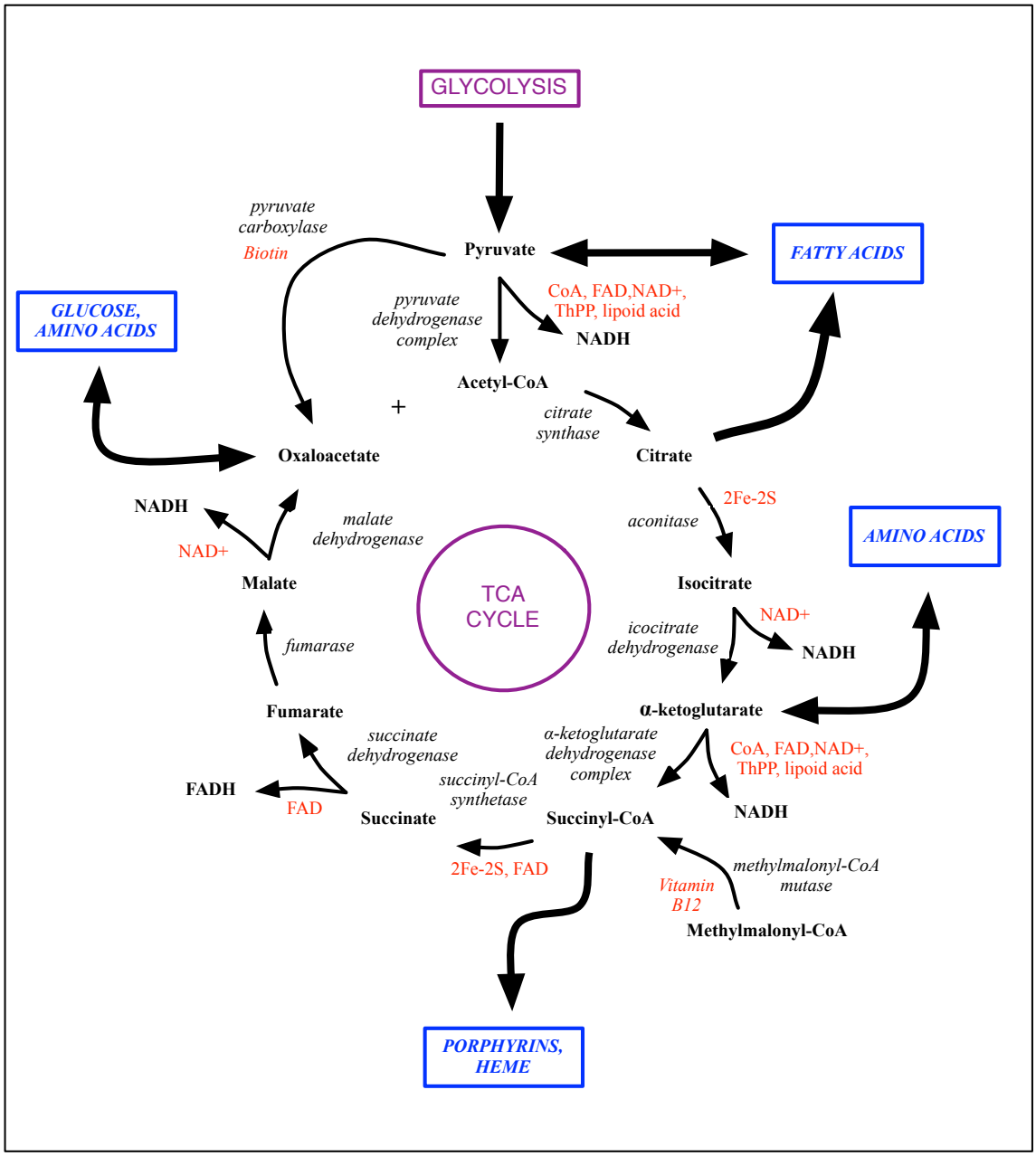

Figure 2.2 The TCA cycle.

Schematic representation of the TCA cycle with major metabolic pathways represented by boxes. Micronutrient cofactors are shown in red at their respective steps. CoA, coenzyme A (pantothenate); ThPP, thiamine pyrophosphate (thiamine); 2Fe-2S; iron-sulfur cluster.

2.2.3 Glycine Synthesis

Glycine is the other initial building block requirement of the heme biosynthetic pathway in humans. It is also important in carbon metabolism and is involved in many anabolic reactions in addition to heme synthesis, including the synthesis of purine nucleotides, glutathione, and creatine. Glycine is not essential in the diet as it is capable of being biosynthesized in a reversible reaction from the amino acid serine. Serine is derived from 3-phosphoglycerate in a two-step reaction requiring an NAD^+ cofactor[67] as well as a pyridoxal 5'phosphate (PLP) cofactor. Glycine formation from serine is catalyzed by serine hydroxymethyltransferase which requires both tetrahydrofolic acid (THFA) and PLP as cofactors. THFA is the active form of folate which can be used in enzymatic reactions and PLP is the active form of vitamin B6. Glycine can also be formed from ammonia, carbon dioxide, and THFA via the "glycine cleavage system" (Figure 2.3).[68] Vitamin B6 deficiency in rats has been shown to result in elevated glycine levels[69] as Vitamin B6 (PLP) is essential in the metabolism of glycine.

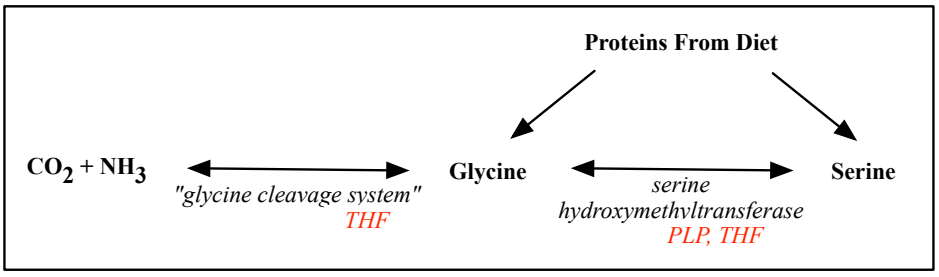

Figure 2.3 Glycine Synthesis from Serine.

2.2.4 Aminolevulinate Synthase

5-Aminolevulinate synthase (ALAS), a homodimer located on the inner mitochondrial membrane,[70] catalyzes the first and rate-limiting step of the heme

biosynthetic pathway through the condensation of succinyl-CoA and glycine to form ALA (Figure 2.4). This is a PLP-dependent reaction. ALAS belongs to the α-oxoamine family of PLP-dependent enzymes.[71] Studies suggest this reaction takes place through an ordered bi uni kinetic mechanism, with glycine binding before succinyl-CoA followed by ALA dissociation after CO_2 and CoA release.[72]

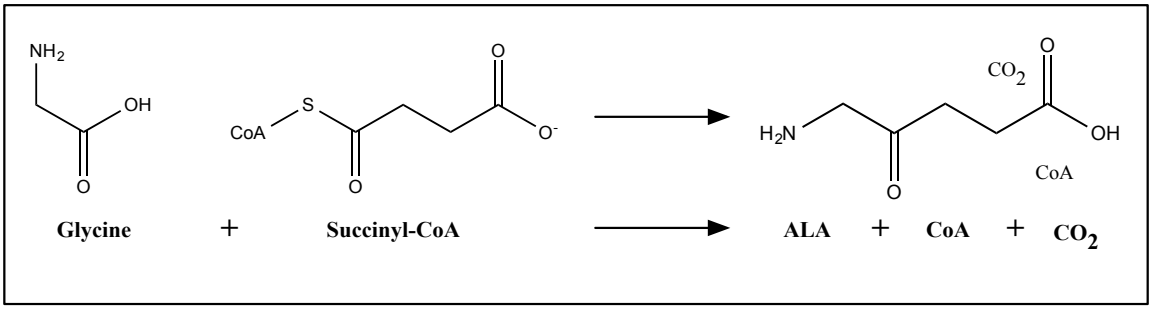

Figure 2.4 Molecular Details of ALAS Reaction.

There are ALAS isoenzymes that are each encoded by separate genes: ALAS1 is the general housekeeping form of the enzyme which is ubiquitously expressed and regulated by the free heme pool; and ALAS2 is the erythroid-specific form which is regulated the iron availability of iron. The heme that is formed in erythrocytes by ALAS2 is utilised primarily for the formation of hemoglobin, whereas heme formed by ALAS1 is dedicated towards myoglobin in muscle cells, cytochromes P450, mitochondrial cytochromes and other hemoproteins in hepatocytes.[3] The regulation of ALAS1 in the liver is sensitive to fluctuations of intracellular heme levels as this rate-limiting enzyme needs to respond rapidly to requirements for heme synthesis. ALAS1 is up-regulated during heme demand, yet when there is sufficient heme, heme inhibits ALAS1 synthesis at both transcriptional and translational steps as well as transfer into mitochondria.[73] In the porphyrias the feedback inhibition of the heme biosynthetic

pathway is disrupted due to the 'bottleneck' of the pathway. This results in up-regulation of ALAS1 and the accumulation of porphyrin precursors.

Studies suggest that ALAS-1 transcription is regulated through the proliferator-activated receptor coactivator 1 (PGC-1),[74] a co-activator of nuclear receptors and other transcription factors. PGC-1 controls mitochondrial biogenesis and oxidative metabolism in many tissues. PGC-1 expression is dampened by insulin and stimulated by glucagon. Elevated PGC-1 levels have been found to cause acute attacks in induced porphyria in mice.[74] This will be discussed further in Section 4.2.1.

Genetic alterations of the ALAS2 gene resulting in under-expression of ALAS2 are known to cause X-linked sideroblastic anemia (XLSA) (Section 3.5.1).[75, 76] Genetic mutations have also been identified that lead to over-expression of ALAS2 activity resulting in a completely different disorder, X-linked dominant protoporphyria (XLPP) (Section 3.4.1).[77-79] A study investigating a different porphyria, congenital erythropoietic porphyria (CEP), caused by a mutation at the third enzyme of the pathway, suggests that specific mutations on the ALAS2 gene increase the severity of this disorder.[80] This indicates that ALAS2 gene mutations may increase the severity of erythropoietic disorders as well as causing XLSA and XLPP.

2.2.5 Aminolevulinate Dehydratase

Aminolevulinate dehydratase (ALAD) is a homooctomer which catalyzes the second step of the heme biosynthetic pathway with the condensation of two molecules of ALA to form PBG (Figure 2.5). Zinc is a required cofactor for enzymatic activity and, as ALAD is an eight-subunit enzyme, this enzyme has the potential to bind eight zinc ions. However, only four zinc ions are required for optimal activity.

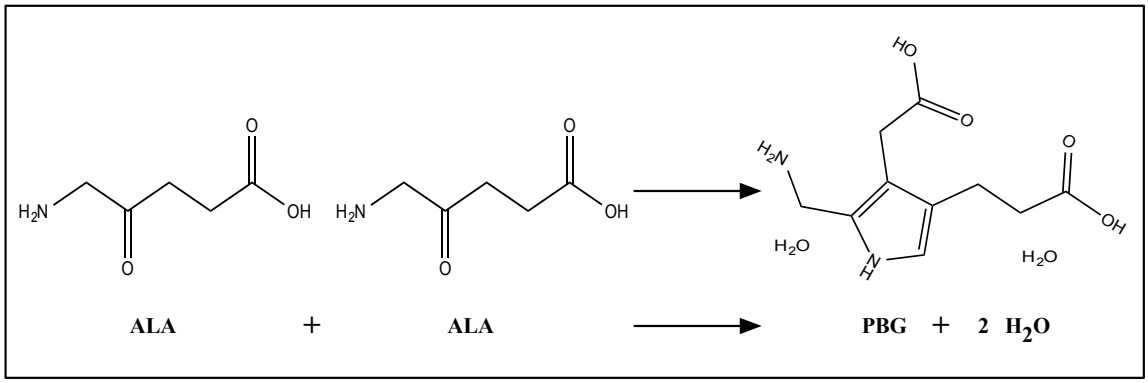

Figure 2.5 Molecular Details of ALAD Reaction.

Mutations of the ALAD gene resulting in diminished ALAD activity cause ALAD-P (Section 3.2.1), a rare form of porphyria which is characterized by the accumulation of ALA. Lead also inhibits this enzyme and can cause lead poisoning symptoms of which resemble ALAD-P (Section 3.5.2). Hepatorenal tyrosemia is another disorder of heme biosynthesis at this step of the heme biosynthetic pathway and presents with accumulation of succinylacetone which inhibits ALAD (Section 3.5.4).[56]

2.2.6 Porphobilinogen Deaminase

Porphobilinogen Deaminase (PBGD) performs the stepwise condensation of four molecules of PBG to form hydroxymethylbilane (HMB), a linear tetrapyrrole (Figure 2.6).[56] This enzyme requires a dipyrromethane cofactor and is also known as hydroxymethylbilane synthase or uroporphyrinogen I synthase. This enzyme is unusual in that its dipyrromethane cofactor is a product of autocatalytic coupling of PBG, the substrate of the reaction.[81] This cofactor acts as a primer for the addition of another four PBG molecules resulting in a linear hexapyrrole. The linear tetrapyrrole (HMB) is then cleaved off leaving the dipyrromethane cofactor still bound to the enzyme.[82]

There is evidence that suggests that folate may also be involved in this reaction as a non-essential activator of PBGD.[83] Folate appears to increase the enzyme's maximum rate of reaction without altering its affinity to PBG.[83]

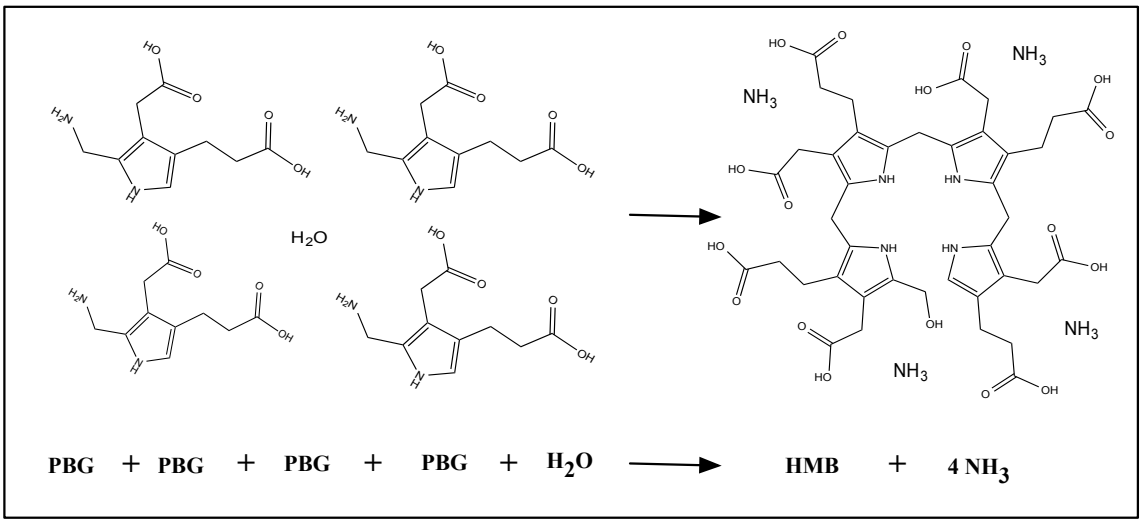

Figure 2.6 Molecular Details of PBGD Reaction.

Like ALAS, there are two forms of PBGD; an erythroid-specific enzyme that may play a regulatory role in heme synthesis and a hepatic enzyme. There does not appear to be any difference in the kinetic parameters of the two isoforms.[84] Defects in PBG-D activity lead to acute intermittent porphyria (AIP), the most common form of acute porphyria (Section 3.2.2).

2.2.7 Uroporphyrinogen III Synthase

Uroporphyrinogen-III synthase (UROS) closes the linear HMB to form the first porphyrin ring of the pathway, uroporphyrinogen III (Figure 2.7). This occurs via cyclisation of HMB to form the first cyclic intermediate of the pathway, uroporphyrinogen III.[85, 86] HMB is unstable and never accumulates *in vivo*. HMB is also capable of spontaneous rapid non-enzymatic cyclisation to form

uroporphyrinogen I, however, there is an excess of UROS capacity in normal physiological conditions, so little of the I isomer forms (Figure 2.8).[3] There is evidence that folate may affect the UROS reaction[87] in addition to PBGD. However, additional studies are required to confirm this.

Diminished activity of this UROS leads to CEP, a porphyria characterized by extreme acute photosensitivity and mechanical skin fragility symptoms (Section 3.4.2).

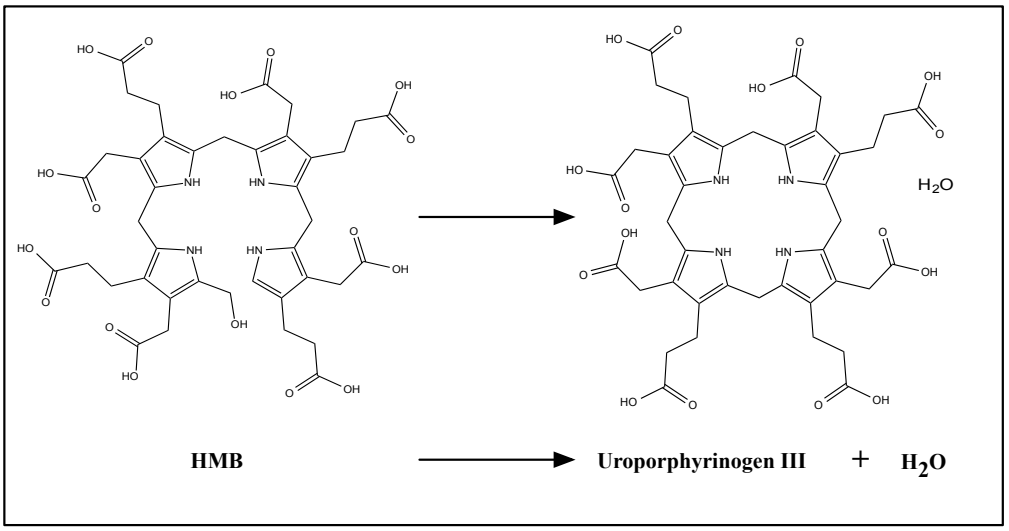

Figure 2.7 Molecular Details of UROS Reaction.

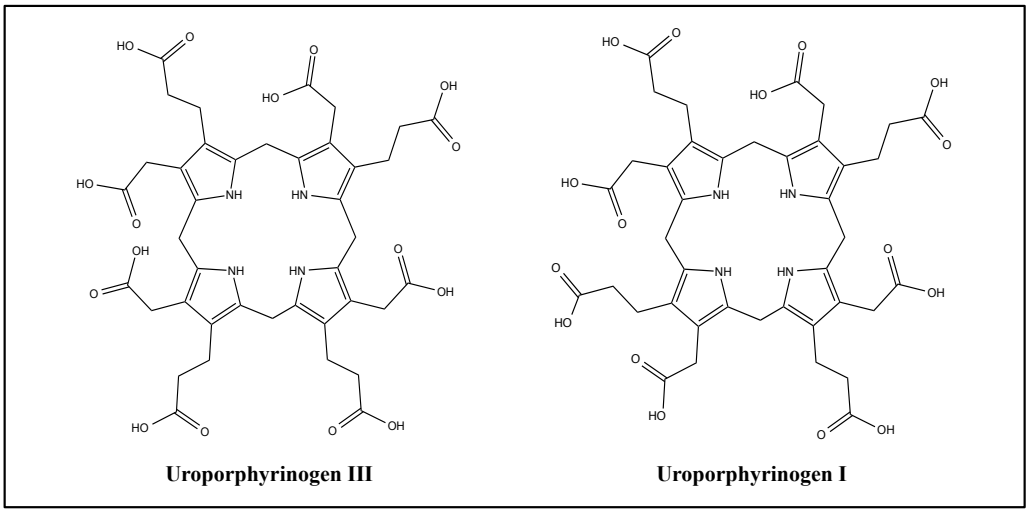

Figure 2.8 Structure Comparison of Uroporphyrinogen isomers.

2.2.8 Uroporphyrinogen Decarboxylase

Uroporphyrinogen Decarboxylase (UROD) is a cytoplasmic enzyme that forms coproporphyrinogen III from uroporphyrinogen III. It is a dimeric enzyme with two single-domain subunits.[56] This enzyme catalyses the sequential decarboxylation of the four acetate substituents of uroporphyrinogen to form coproporphyrinogen. This reaction occurs via hepta-, hexa-, and penta-carboxylate intermediate forms (Figure 2.9) [88] which are normally detected in small amounts in the urine, liver, and plasma.[3] This reaction appears to occur independently of cofactors or metal ions although its activity does appear to be enhanced by lipoic acid.[89] UROD also metabolises uroporphyrinogen I to coproporphyrinogen I, although this metabolite plays no further role in the pathway.

Mutations of this UROD may result in porphyria cutanea tarda (PCT) or hepatoerythropoietic protoporphyria (HEP) both of which present with characteristic symptoms of acute photosensitivity, mechanical skin fragility, and liver damage (Section 3.3). PCT is the most common form of porphyria.

2.2.9 Coproporphyrinogen-III Oxidase

Coproporphyrinogen-III oxidase (CPOX) catalyses the oxidative decarboxylation of coproporphyrinogen III to produce protoporphyrinogen IX (Figure 2.10). CPOX is a mitochondrial enzyme located in the inter-membrane space. This enzyme contains two internally bound Fe^{2+} ions and requires the presence of molecular oxygen as an electron acceptor. Copper ions may also play a role in this step as an essential activator[90, 91] although other studies suggest otherwise.[92] This enzyme does not utilise

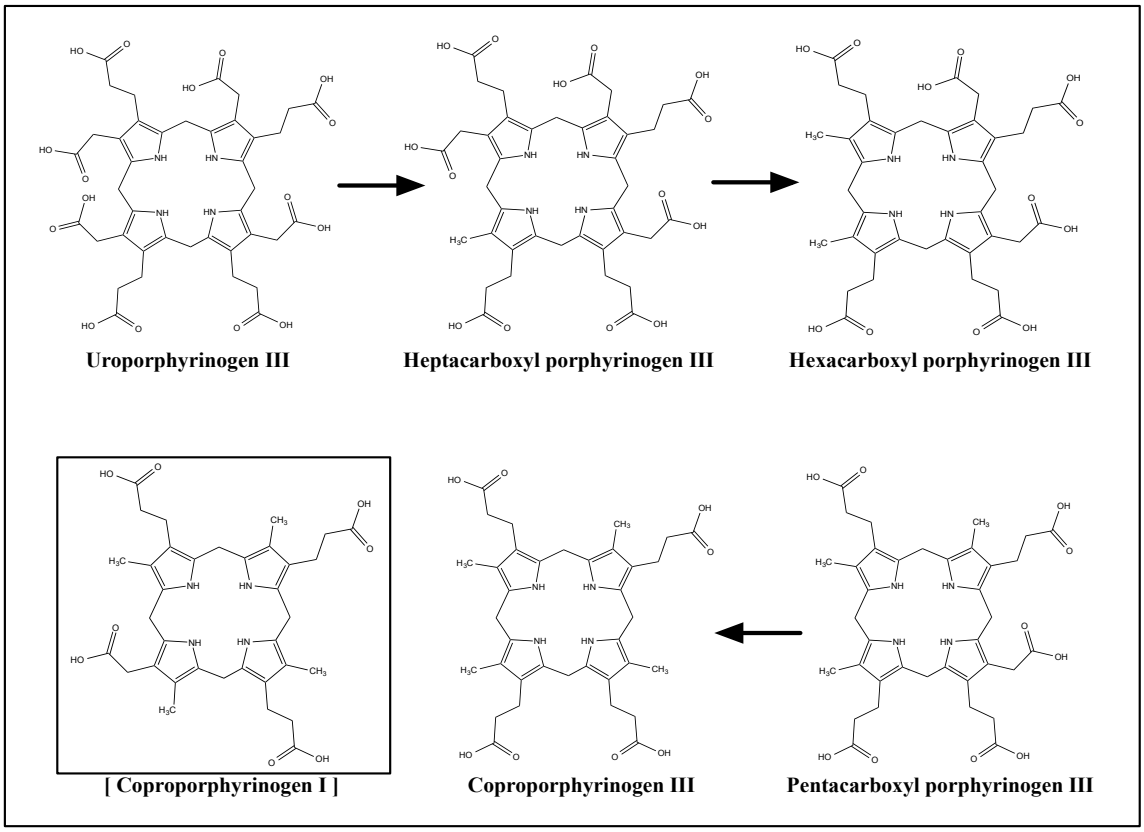

Figure 2.9 Coproporphyrinogen and Its Intermediates.

The intermediary forms of coproporphyrinogen synthesis are shown as well as the coproporphyringogen I isomer which plays no further role in the heme biosynthetic pathway.

coproporphyrinogen I as a substrate and the I isomer plays no further role in the heme biosynthetic pathway.

Diminished enzymatic activity of CPOX results in the acute porphyria, hereditary coproporphyria (HCP) (Section 3.2.3). This is a mixed porphyria that can present with both the acute symptoms of AIP and the skin symptoms of the cutaneous porphyrias. HCP presents with symptoms similar to AIP (mutation at PBGD) due to inhibition of the enzyme PBGD by coproporphyrinogen (Figure 2.1).[93]

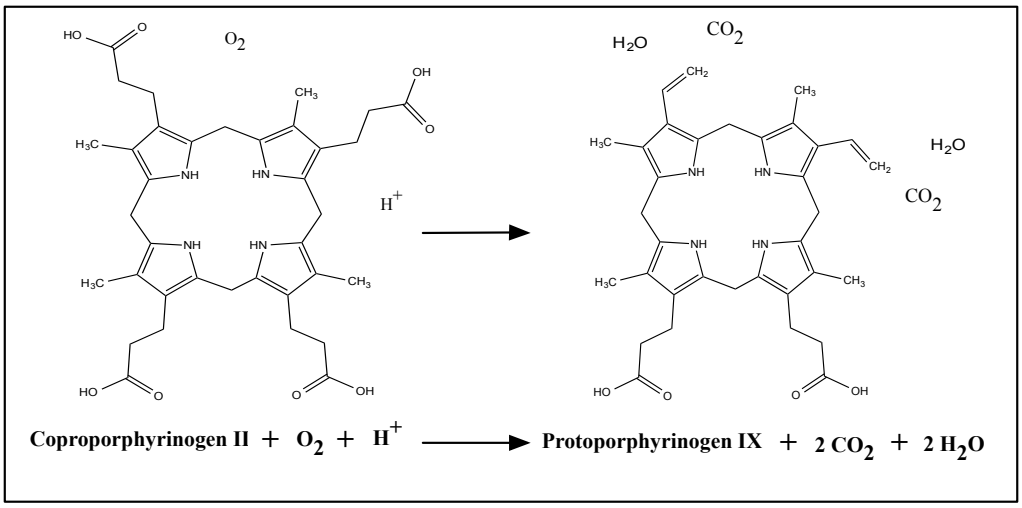

Figure 2.10 Molecular Details of CPOX Reaction.

2.2.10 Protoporphyrinogen Oxidase

Protoporphyrinogen oxidase (PPOX) synthesizes protoporphyrinogen IX from protoporphyrin IX in an oxidation reaction (Figure 2.11). This enzyme is a homodimer located on the outer surface of the mitochondria membrane and utilises an FAD cofactor which transfers electrons from the substrate to the electron acceptor oxygen.[85, 94, 95]

Diminished activity of PPOX results in VP which can present with acute symptoms similar to those in AIP due to the negative feedback of the precursors protoporphyrinogen and coproporphyrinogen on PBGD, the enzyme deficient in AIP (Figure 2.1).[93]

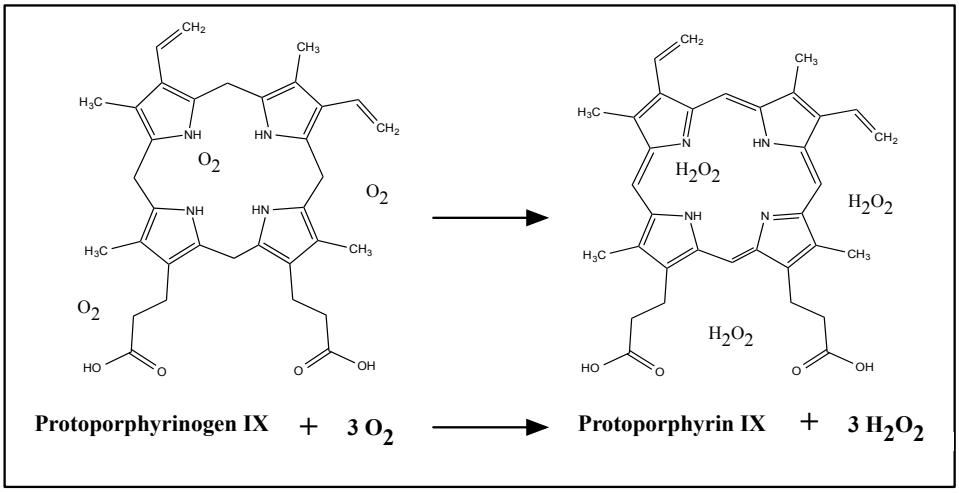

Figure 2.11 Molecular Details of PPOX Reaction.

2.2.11 Ferrochelatase

Ferrochelatase (FECH) catalyzes the final step of heme formation, the insertion of the ferrous form of iron into protoporphyrin IX to form heme, the end product of the pathway (Figure 2.12). FECH requires both iron and copper for activity and utilizes an iron-sulfur cluster as a cofactor. Copper stimulates the activity of FECH and increases its affinity for iron. It has been suggested that PLP may also act as a coenzyme or non-essential activator of FECH[96, 97] although this has not been confirmed. This enzyme is dimeric with each subunit consisting of similar domains and is located in the mitochondrial inner membrane. Diminished activity of this enzyme results in the cutaneous porphyria, erythropoietic protoporphyria (EPP) (Section 3.4.3).

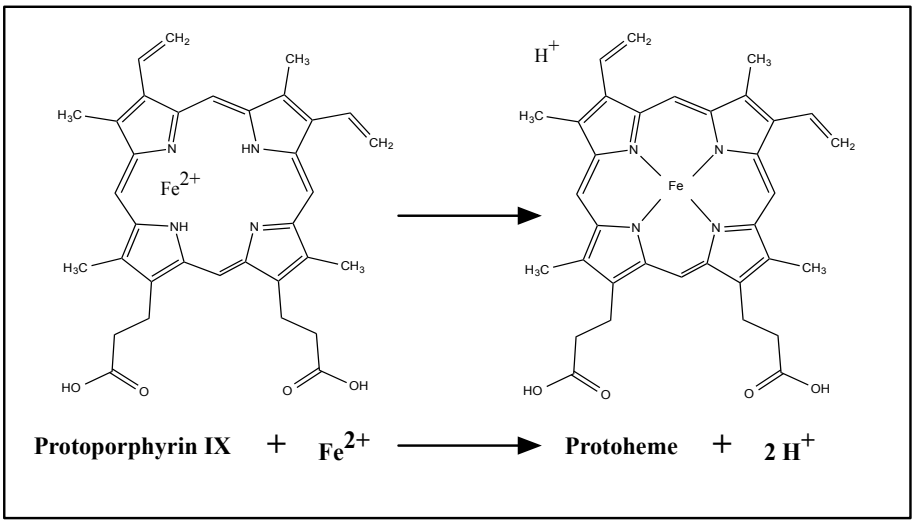

Figure 2.12 Molecular Details of FECH Reaction.

2.2.12 Heme Protein Utilisation and Degradation

2.2.12.1 Heme Proteins

Heme can be utilised to make up a variety of essential enzymes and thus carries out many diverse functions through heme-binding proteins.[3] These include mitochondrial cytochromes that assist the stepwise transport of electrons down electrochemical gradients in electron transport and coupled oxidative phosphorylation including: cytochrome oxidases, cytochromes P450, tryptophan pyrrolase and deoxygenases. Heme is also a constituent of nitric oxide synthase, myeloperoxidase, and thyroid peroxidase which are all involved in oxidation reactions, as well as catalase which assists the decomposition of hydrogen peroxidase. Heme can also function as a redox sensor where it affects the activity of nuclear receptors and other proteins in signaling and metabolism. The majority of heme, however, goes to haemoglobin, myoglobin, and neuroglobin which transport oxygen to, and carbon dioxide from, tissues.

Synthesis of heme in erythrocytes accounts for 85% of the total daily heme production[73] and is utilised in hemoglobin for oxygen transport between the lungs and

tissues.[85] The remaining 15% of daily heme production occurs in the liver and results in the formation of heme-based enzymes. In hepatocytes, 65% of the heme formed is utilized in cytochrome P450 enzymes.[98] These enzymes are important for the detoxification of foreign substances. When cytochrome P450 enzymes are induced, ALAS1 is up-regulated to meet heme requirements. This is significant in the porphyrias as the up-regulation of ALAS1 can result in over-production of heme precursors when there is deficient enzyme activity at one or more of the steps of the heme biosynthetic pathway. Drugs requiring cytochrome P450 detoxification pathways are known to aggravate the porphyric state. Of the remaining heme formed in hepatocytes, about 15% is utilised for the synthesis of catalase which is localised in peroxisomes, about 8% for synthesis of cytochromes b5, and about 6% goes to formation of mitochondrial cytochromes.[98]

2.2.12.2 Heme Degradation

Cellular heme levels are tightly regulated through balancing heme biosynthesis and catabolism.[63] Heme is broken down by heme oxygenase, the first and rate-limiting enzyme of heme degradation, into biliverdin. Biliverdin reductase then transforms biliviridin into bilirubin which is excreted as a bile pigment. Both of these reactions require nicotinamide adenine dinucleotide phosphate (NADPH).[99] Biliverdin and bilirubin are both antioxidants capable of protecting mammalian tissues and cells from oxidative stress.

2.3 Micronutrient Cofactors

Through a brief summary of heme biosynthesis, it is apparent that the biosynthesis of heme involves a variety of micronutrient cofactors including vitamin B6, riboflavin, biotin, pantothenic acid, lipoic acid, zinc, iron, and copper (Table 2.1). These essential

micronutrients that are involved in heme biosynthesis will be briefly discussed, notably their functions as cofactors and the corresponding implications for the porphyrias.

2.3.1 B-vitamins and Heme Synthesis

The physiological interactions between the B vitamins are complex.[100] The B-vitamins are essential to myriad biosynthetic and metabolic pathways. B-vitamin deficiencies have been reported to correlate with increased porphyrin production[101] suggesting a connection to the heme biosynthetic pathway and thus the porphyrias. The B-vitamins are tightly interconnected through their metabolic pathways and altering levels of one B-vitamin will result in changes to the levels of others.[100] Some of the B vitamin interrelationships and interdependencies are: the reliance of niacin (nicotinic acid) formation from tryptophan on the participation of vitamin B6 (PLP) as well as riboflavin; riboflavin is required for vitamin B12 synthesis; riboflavin and niacin (nicotinic acid) are both required for vitamin B6 synthesis; vitamin B6 deficiency results in impairment of vitamin B12 absorption;[102] biotin deficiency and pantothenate deficiency aggravate one another.[100] The complex and tightly regulated interdependencies between the B-vitamins may play a significant role in the porphyrias if the depletion of one B-vitamin contributes to the depletion of other B-vitamins. This can result in a cascading detrimental metabolic effect.

Table 2.1 Micronutrients Involved in Heme Biosynthesis Pathways.

Nutrient	Pathway	Enzyme
Thiamine (ThPP)	TCA cycle	Pyruvate dehydrogenase complex
		α-Ketoglutarate dehydrogenase complex
Riboflavin (FAD)	TCA cycle	Pyruvate dehydrogenase complex
		α-Ketoglutarate dehydrogenase complex
		Succinyl-CoA synthetase
		Succinate dehydrogenase
	HBP	PPOX
Niacin (NAD+)	TCA cycle	Pyruvate dehydrogenase complex
		α-Ketoglutarate dehydrogenase complex
		Isocitrate dehydrogenase
		Malate dehydrogenase
Pantothenate (CoA)	TCA cycle	CoA synthetase
Vitamin B6 (PLP)	HBP	ALAS, FECH
	Glycine synthesis	Serine hydroxymethyltransferase
Biotin	TCA cycle	Pyruvate carboxylase
Folate (THFA)	Glycine synthesis	Serine hydroxymethyltransferase
		"Glycine cleavage system"
	HBP	PBGD, UROS
Vitamin B12	TCA cycle	Methylnalonyl-CoA mutase
Lipoic Acid	TCA cycle	Pyruvate dehydrogenase complex
		α-Ketoglutarate dehydrogenase complex
	HBP	UROD
Iron (Fe^{2+})	TCA cycle	Aconitase
		Succinyl-CoA synthetase
	HBP	CPOX
		FECH
Copper (Cu^{+})	HBP	FECH
Zinc (Zn^{2+})	HBP	ALAD

Nutrients known to be involved in heme biosynthetic pathway (HBP) enzymes either directly or through the synthesis of the substrates succinyl-CoA (TCA cycle) or glycine (glycine synthesis).

2.3.1.1 Thiamine

Thiamine (vitamin B1) is an essential nutrient in humans and, as previously mentioned, is a prosthetic group of ThPP. ThPP acts as a coenzyme for several biochemical reactions including that of the pyruvate dehydrogenase complex for pyruvate synthesis and the alpha-ketoglutarate dehydrogenase complex for succinyl-CoA synthesis in the TCA cycle. Serotonin metabolism may be affected by the porphyrias (Section 5.3.2) and cerebral serotonin uptake may be disturbed by thiamine deficiency[103] leading to elevated serotonin levels in some parts of the brain.[104] Elevated serotonin levels can result in serotonin syndrome[105] and the symptoms of serotonin syndrome correspond to many of the symptoms of acute porphyria.[91] This suggests that thiamine deficiency may play a role in the elevated serotonin levels seen in the acute porphyrias.

2.3.1.2 Riboflavin

Riboflavin (vitamin B2) is the water-soluble precursor of two flavin coenzymes, flavin mononucleotide and FAD.[106] FAD is involved in a variety of important metabolic reactions including four enzymatic processes of the TCA cycle as well the protoporphyrinogen oxidase reaction of the heme biosynthetic pathway (Figures 2.1 and 2.2). Riboflavin deficiency leads to increased urinary coproporphyrins that can reach pathological levels[101] and has been reported in a case of HCP.[8] HCP can present with skin symptoms (Section 3.2.3). Deficiency of riboflavin affects collagen content and crosslinking in the skin of rats impairing wound healing.[107, 108] It is known that high levels of porphyrin in erythrocytes result in the cutaneous symptoms of porphyria. Could riboflavin deficiency also contribute to the pathogenesis of the skin

symptoms of the cutaneous porphyrias? Riboflavin deficiency leads to a vitamin B6 deficiency as well, due to riboflavin's role in vitamin B6 synthesis.[100]

2.3.1.3 Niacin

Niacin (vitamin B3) is another essential nutrient in the human body. Niacin in the form of NAD^{+}[109] is an essential cofactor involved in the TCA cycle. This important coenzyme is found in all living cells and is involved in redox reactions as a hydride transfer agent. It can also be synthesized from the amino acid tryptophan in humans (Section 5.3.2).[109] Niacin deficiency results in pellagra; symptoms of pellagra are complex and involve the skin, digestive tract, and nervous system.[110] Pellagra patients also excrete increased amounts of coproporphyrins in the urine[101] suggesting a connection to heme biosynthesis malfunction. Deficiency of this micronutrient may also play a role in porphyria symptoms.

2.3.1.4 Pantothenate

Pantothenate (vitamin B5) is also an essential nutrient and is required as a constituent of CoA. Synthesis and oxidation of fatty acids rely on CoA[111] as do the pyruvate dehydrogenase complex and the alpha-ketoglutarate dehydrogenase complex of the TCA cycle (Figure 2.2). Pantothenate is connected to porphyrin metabolism through succinyl-CoA (Section 2.2.2).[112] Excess porphyrins have been found excreted in the tears of pantothenate-deficient rats.[113] This suggests that pantothenate deficiency may result in an altered heme biosynthetic pathway. Further studies would be required to investigate this possible connection.

2.3.1.5 Vitamin B6

Vitamin B6 in its active form of PLP is an essential cofactor in over 300 enzymatic reactions including many involved either directly or indirectly in heme synthesis. It acts as a coenzyme in transamination reactions as well as decarboxylation and deamination reactions of amino acids. PLP plays a major role in the heme biosynthetic pathway as it is an essential cofactor of the rate-limiting ALAS enzyme of heme synthesis (Figure 2.1), mutations of which cause XLSA among other disorders. One-third of patients with XLSA are pyridoxine responsive,[114, 115] and ALAS2 mutations of this disorder are commonly observed in or near the PLP-binding site of the ALAS2 enzyme.[116] Pyridoxine is a form of vitamin B6 and is converted to the active coenzyme PLP. This illustrates the importance of PLP in the ALAS reaction.

Vitamin B6 deficiency has been found to stimulate increased iron deposits.[117] Increased iron deposits are commonly found in porphyria and lead to increased oxidative damage. Other important PLP reactions are the conversion of tryptophan to both niacin and serotonin. Vitamin B6 is also important in the maintenance of Na^+ and K^+ balance[118] as well as Ca^{2+} and Mg^{2+} balance.[119] In addition, vitamin B6 deficiency is shown to impair vitamin B12 absorption in rats resulting in a secondary vitamin B12 deficiency in addition to the primary vitamin B6 deficiency.[102] The peripheral neuropathy resulting from a vitamin B6 deficiency is similar to that found in the porphyrias.[120-122]

2.3.1.6 Biotin

Biotin (vitamin B7) plays an important role as a cofactor in the metabolism of carbohydrates, lipids, and proteins[123] as well as in the regulation of genetic expression of mitochondrial carboxylases in rats.[124] It is a cofactor for pyruvate carboxylase, propionyl-CoA carboxylase, beta-methylcrotonyl-CoA carboxylase and acetyl-CoA carboxylase. These biotin-dependent carboxylases are found in the mitochondria where they replace intermediates in the TCA cycle that are regularly removed to feed other processes.[125] In biotin deficient states, methylcrotonyl-CoA accumulates in the mitochondria and reacts with glycine, which may lead to glycine depletion. Deficiency of biotin may therefore reduce both succinyl-CoA and glycine levels due to diminished enzymatic activity of biotin-dependent enzymes. This could affect heme biosynthesis as succinyl-CoA and glycine are the initial building blocks of the heme biosynthetic pathway.

2.3.1.7 Folate

Folate (vitamin B9) is an essential cofactor in DNA synthesis and repair as well as acting as a cofactor in many other biological reactions. Its biologically active form is THFA, which is involved in the metabolism of amino acids as well as nucleic acids. Serine hydroxymethyltransferase is a THFA-dependent enzyme that provides a route to glycine synthesis through the reversible conversion of serine to glycine (Figure 2.3). Folate may also act as a non-essential activator of PBGD,[83] the enzyme with reduced activity in AIP. There is also evidence that folate activates uroporphyrinogen III synthetase, the enzyme that has reduced activity in CEP.[87] Folate metabolism is connected with vitamin B12 metabolism and deficiencies of either of these vitamins

can result in severe neurological and psychiatric disturbances.[126] Some of these symptoms are similar to those found in the porphyrias.

2.3.1.8 Vitamin B12

Vitamin B12 (cobalamin) is a cofactor in the conversion of homocysteine to methionine and the conversion of methylmalonyl-CoA to succinyl-CoA. A vitamin B12 deficiency alters levels of other B-vitamins with lowered riboflavin and vitamin B6 levels as well as elevated folate levels.[127] Levels of all B vitamins returned to normal with vitamin B12 supplementation.[127] The methylcobalamin form of vitamin B12 may also induce increased melatonin production. Melatonin acts as an important antioxidant in the porphyrias.[128-132] Deficiency of vitamin B12 can have severe manifestations[126, 133] including peripheral neuropathy, seizures, and paralysis; symptoms that can also be found in the acute porphyrias.

2.3.2 Other Micronutrients in Heme Synthesis

In addition to the B vitamins, the micronutrients lipoic acid, copper, iron and zinc are also essential to heme biosynthesis.

2.3.2.1 Lipoic Acid

Lipoic acid is an important cofactor in a variety of enzyme complexes and is an essential cofactor of the TCA cycle. Lipoic acid supplementation has been shown to improve cognitive functioning[134] and acts as an antioxidant, reversing iron-induced oxidative stress.[108] UROD activity, deficiency of which causes PCT, has been suggested to be enhanced by lipoic acid possibly through activation as a non-essential cofactor.[89] It is suggested that supplementation with lipoic acid alleviates some of the

symptoms of PCT through its free-radical scavenging ability and through directly enhancing the activity of uroporphyrinogen decarboxylase.

2.3.2.2 Iron

Iron-sulfur clusters are cofactors in several enzymes relating to the heme biosynthetic pathway including FECH, the final enzyme in the pathway, as well as two reactions of the TCA cycle. The formation of iron-sulfur clusters from cysteine desulfurase is a PLP-dependent reaction.[135] Deficiencies of iron, PLP, sulfides or loss of enzymatic activity could result in a failure to produce iron-sulfur clusters.[136, 137] Along with heme, iron-sulfur clusters are essential for the assembly and optimal activity of electron transfer complexes where they act as prosthetic groups. Iron-sulfur clusters are involved in reducing oxygen to water which generates the proton gradient across the inner mitochondrial membrane for ATP production. ATP production is essential for the normal function of every cell and processing of iron into heme and iron-sulfur clusters is important for both mitochondria maintenance and ATP production. Lack of synthesis of either heme or iron-sulfur clusters can result in massive oxidative damage in mitochondria, cells, and tissue.[138] Could this play a role in the symptomology of the porphyrias?

2.3.2.3 Copper

Copper is involved in electron transport as well as oxygen transport. Copper has been suggested to stimulate both CPOX[90] and FECH activity in the heme biosynthetic pathway (Table 2.1). Deficiency of copper results in impaired utilization of iron for hemoglobin production[139] and thus increased dietary iron absorption.[140] Deficient

FECH activity is the underlying factor in EPP making adequate copper concentrations a potential concern in patients with this porphyria.

2.3.2.4 Zinc

Zinc is an essential trace element that is involved in almost 100 known enzymatic reactions.[141] Zinc deficiency results in oxidative damage to DNA as well as inactivation of zinc-containing proteins.[142, 143] It is a required cofactor for the conversion of ALA to PBG by ALAD, with four zinc ions needed for optimal activity. Zinc deficiency has been suggested to be a factor in some of the symptoms of porphyria[144] although little supporting data is present. However zinc has been reported to successfully treat AIP patients resulting in pain relief.[26, 27, 145] This suggests that zinc may indeed play a role in the symptomology of the acute porphyrias.

2.4 Summary of Chapter 2

The heme biosynthetic pathway is of paramount importance to understanding the porphyrias due to the diminished activity of one or more enzymes of this pathway being a fundamental requirement in the manifestation of these disorders. The key to understanding the porphyrias lie not only in an understanding of heme biosynthesis but also of the many interconnected and related biochemical pathways. The biochemical pathways that are impacted by disordered heme metabolism are wide-reaching and can affect every system of the body. The dependence of the heme biosynthetic pathway on a vast array of micronutrients and the complex interrelationships and dependencies of these micronutrients on each other highlight the biochemical intricacies of heme biosynthesis. A thorough examination of these

pathways may lead to an explanation of the biochemical mechanisms underlying the symptomology of the porphyrias and offer insights into rational treatments and prophylaxis of these disorders.

CHAPTER 3. THE PORPHYRIAS

3.1 Background

The porphyrias are a group of inherited metabolic disorders involving diminished activity of one or more of the enzymes of the heme biosynthetic pathway (Figure 2.1). As previously described, this diminished enzyme activity results in a hyper-metabolic state prior to the partial block(s) caused by the diminished enzyme activity(ies). This 'bottleneck' in the heme pathway results in excess accumulation of precursors before the affected enzyme and abnormal biochemical precursor patterns. Each porphyria presents with a unique precursor accumulation pattern (Table 3.1) depending on where the 'bottleneck' in the pathway occurs. The increased activity of the heme biosynthetic pathway is due to up-regulation of the initial rate-controlling enzyme, ALAS, as the pathway continues to attempt to meet the demand for heme.

The porphyrias are generally classified in one of two ways: (1) as hepatic or erythropoetic depending on the main site of precursor accumulation, or (2) as acute or cutaneous depending on the predominant symptoms (Table 3.1).

The acute porphyrias are characterized by acute neurovisceral attacks and can present with a wide variety of symptoms leading acute porphyria to have been termed the "little imitator".[146] Acute attacks can be triggered by a number of factors including drugs, chemicals, hormones, fasting, sun exposure, infections and exercise. Some of the symptoms that can present in acute porphyria include abdominal pain, autonomic instability, and mental disturbances. Between 70-90% of the carriers of a genetic mutation associated with an acute porphyria remain latent or asymptomatic and may never present with symptoms.[147, 148] For the discussions in the following chapters the

term ‘porphyria’ and any of the specific porphyria abbreviations will assume the symptomatic form of the disorder and ‘latent’ will be used to refer to the asymptomatic form. The acute porphyrias include AIP (acute intermittent porphyria), VP (variegate porphyria), HCP (hereditary coproporphyria), and ALAD-P (aminolevulinate dehydratase deficiency porphyria). VP and HCP can present with cutaneous symptoms as well as acute symptoms (Table 3.1) and have been referred to as mixed porphyrias as a result.

The cutaneous porphyrias are characterized by varying degrees of photosensitivity with resulting skin damage and include PCT (porphyria cutanea tarda), HEP (hepatoerythropoietic porphyria), EPP (erythropoietic protoporphyria), CEP (congenital erythropoietic porphyria), and the recently described XDPP (X-linked dominant erythropoietic protoporphyria). Other disorders involving abnormalities of the heme pathway include XLSA (X-linked sideroblastic anemia), iron deficiency anemia, tyrosemia, and lead poisoning.

Most of the acute porphyrias are inherited in an autosomal dominant pattern although the rare ALAD-P is inherited in a recessive pattern (Table 3.1). There are reports of cases of the common acute porphyrias being inherited in different patterns as well, e.g. a case of VP inherited in an autosomal recessive pattern.[149] The majority of the cutaneous porphyrias are inherited in an autosomal recessive fashion (Table 3.1). There have also been reports of two types of porphyria in one family,[150-152] as well as two types of porphyria in one patient (dual porphyria).[152-155] The latter is usually a combination of AIP and VP (Chester Porphyria) or a combination of PCT and VP. Other reported dual porphyrias include deficiencies of both CPOX and ALAD activity in one patient[156] and the co-existence of HCP and PCT in another.[157]

3.2 The Acute Hepatic Porphyria's

There are six hepatic porphyrias: AIP, VP, HCP, ALAD-P, PCT, and HEP. Of these only four are considered acute: AIP, VP, HCP, and ALAD-P (Table 3.1). The "acute attack" characteristic of the acute porphyrias usually manifests with severe pain that is most often abdominal (although sometimes peripheral) along with central nervous system (CNS) dysfunction. As mentioned above, the genetic defect in these porphyrias is necessary but not sufficient to cause acute attacks as most patients with the mutation remain latent and may never experience an attack.[147, 148] An external factor is required to trigger the symptoms including but not limited to drugs and chemicals that induce cytochrome P450 enzymes, enzymes which require a heme prosthetic group,[158, 159] surgery, stress, alcohol consumption, smoking, fasting, exercise, and hormonal changes.

As mentioned earlier, an external factor as well as the inherited enzyme deficiency is necessary to cause an acute attack. Anderson suggested that 90% of those with an inherited mutation may never experience an acute attack.[160] The external precipitating factors are thought to put strain on the heme pathway through induction of heme-containing enzymes and hepatic ALAS1. Cytochrome P450-inducing drugs and chemicals are arguably the most common contributing factors that trigger an acute attack. Many drugs are metabolized and detoxified through cytochrome P450 enzymes which contain a heme prosthetic group. Therefore, when exposed to a drug that is metabolized via the cytochrome P450 pathways, heme demands are increased resulting in ALAS1 induction at the beginning of the heme biosynthetic pathway. This activity at the beginning of the heme biosynthetic pathway is exaggerated due to

a partial block of the heme pathway at PBGD resulting in the elevated precursors.[161, 162]

Endocrine factors can also trigger an acute attack; AIP is more common in women than men and usually presents after puberty. Some women experience premenstrual porphyrinogenic symptoms every month and acute attacks are less common after menopause. Oral contraceptives and other exogenous steroids can trigger acute attacks. Pregnancy has also been reported to provoke porphyria,[163] although pregnancy appears well tolerated in a review of pregnancies in acute porphyria patients.[164] Kauppanin and Mustajoki[164] conducted a study including 76 women with AIP and VP and 176 pregnancies. 92% of these patients reported no porphyria symptoms during pregnancy. If pregnancy does not usually trigger severe attacks,[3] could this be due to avoidance of harmful drugs and optimised nutritional status during pregnancy?[164] Diet and nutritional status are important contributors to porphyria, but they are not well studied as accurate dietary histories are often difficult to obtain. However, low carbohydrate diets and fasting are known to trigger acute attacks, and glucose is a common treatment of acute attacks through suppression of ALAS1 (see Chapter 4).[165] Optimal micronutrient status is also likely to play an important role in the porphyrias due to the reliance of heme biosynthesis on so many micronutrient cofactors (Table 2.1).

Characteristic of the acute hepatic porphyrias is increased excretion of the porphyrin precursors ALA and PBG due to diminished activity of ALAD or PBGD. In AIP this occurs genetically through mutations of PBGD and in VP and HCP through inhibition of PBGD by the accumulation of protoporphyrinogen and/or coproporphyrinogen (Figure 2.1).[93] In ALAD-P it is decreased activity of ALAD rather than PBGD that

causes the altered heme metabolism resulting in ALA buildup with normal PBG levels (Table 3.1). The pathophysiology of the acute attack is not fully understood and will be discussed in Chapter 5. Suggested mechanisms include accumulation of neurotoxic porphyrin precursors (e.g. ALA), deficiency of heme leading to altered metabolism in heme-dependent pathways, and depletion of essential cofactors and substrates (e.g. PLP, folate, zinc).[73] Management of the acute porphyrias will be discussed in Chapter 4.

The manifestations of the acute symptoms of the different acute porphyrias are similar in presentation although AIP tends to be the most severe.

3.2.1 ALAD Deficiency Porphyria (ALAD-P)

ALAD-P is a very rare porphyria, with less than 10 cases reported worldwide. ALAD-P is characterized by depressed ALAD activity (>90%) and can manifest with homozygous[167, 168] and compound heterozygous cases. Lead depresses ALAD activity and lead poisoning presents with the same symptoms as ALAD-P. Unlike the other acute hepatic porphyrias which have been successfully reversed with liver transplants, as will be described in Section 4.4, a case of a liver transplant in ALAD-P resulted in persisting acute symptoms suggesting this porphyria may originate in other tissues in addition to hepatocytes.[169] Clinically ALAD-P mimics AIP (discussed below), however, it results in increased ALA and coproporphyrin levels in the urine but with normal PBG levels (Table 3.1). As ALAD-P is rare with only a few

Table 3.1 Classification and Precursor Patterns of the Porphyrias.

Porphyria	Deficient enzyme	Inheritance Pattern	Major symptoms	Enzyme activity % of normal	Erythrocytes	Urine	Stool
ALAD-P	ALAD	AR	NV	~ 5	Zn-protoporphyrin	ALA, coproporphyrin III	—
AIP	HMBS	AD	NV	~ 50	---	ALA, PBG, uroporphyrin	—
HCP	CPOX	AR	NV + CP	~ 50	—	ALA, PBG coproporphyrin III	coproporphyrin III
VP	PPOX	AD	NV + CP	~ 50	—	ALA, PBG, coproporphyrin III	coproporphyrin III, protoporphyrin
PCT	UROD	AD	CP	< 20	uroporphyrin 7-carboxylate porphyrin	uroporphyrin 7-carboxylate-porphyrin	isocoproporphyrin
HCP	UROD	AR	CP	< 10	Zn-protoporphyrin	uroporphyrin 7-carboxylate-porphyrin	coproporphyrin
CEP	UROS	AR	CP	~ 1 - 5	uroporphyrin coproporphyrin 1	uroporphyrin I coproporphyrin I	coproporphyrin I
EPP	FECH	AR	CP	~ 20-30	free protoporphyrin	—	protoporphyrin
XLP	ALAS2	XL	CP	> 100	Zn-protoporphyrin	—	protoporphyrin

Table based from Balwani et al. 2012.[166] The top highlighted band reports the acute hepatic porphyrias, the middle band reports the chronic hepatic porphyrias and the bottom band reports the cutaneous erythropoietic porphyrias. NV; neurovisceral, CP; cutaneous photosensitivity, AR; autosomal recessive, AD; autosomal dominant, XL; X-linked.

confirmed cases worldwide, severity and age of onset as well as biochemical mechanisms and treatment have not been well studied.

3.2.2 Acute Intermittent Porphyria (AIP)

Genetic mutations resulting in diminished PBG-D activity lead to AIP, the most common as well as most severe form of acute porphyria. Those with this genetic defect generally experience good health but are at risk of developing an acute attack. These attacks be life threatening and can vary in duration from several days up to several months.

It is the effects on the nervous system that appear to lead to most of the clinical features of AIP. Symptoms of AIP are non-specific and highly variable making AIP easily confused with other diseases including appendicitis, pancreatis, and gallstones. This porphyria has been termed the 'little imitator' due to its ability to mimic and be easily misdiagnosed as many other diseases. Acute attacks of AIP can present with any one or a combination neurovisceral dysfunction characterized by the classic triad of symptoms: excruciating abdominal pain; peripheral neuropathy and psychiatric disturbances. Symptoms can include weakness, paraesthesiae, numbness, constipation, nausea, vomiting, tachycardia, hypertension, seizures, fever, anxiety, and psychosis.[73, 170] Seizures can be challenging to treat as the most common anti-epileptics are porphyrinogenic; exacerbating an acute attack. Mortality of AIP was three times that of the general population according to a study in 1996,[171] and death is usually the result of respiratory paralysis from trunk muscle weakness.[172] Porphyria is most dangerous when undiagnosed as porphyrinogenic drugs can be inadvertently prescribed resulting in worsening symptoms or death.

AIP is inherited in an autosomal dominant pattern with the onset of symptoms usually after puberty. Initial diagnosis is through urinary ALA and PBG levels. AIP patients present with elevated ALA and PBG levels in the urine but with normal erythrocyte and fecal porphyrins (Table 3.1), ALA and PBG levels are elevated further during acute attacks. Urine is often recorded as pink, brown, dark red, and even black with exposure to air and light.[3, 173, 174] This is interesting as ALA and PBG, the elevated precursors in this disorder, are colourless. However, PBG can be non-enzymatically converted into uroporphyrin and as a result uroporphyrin and coproporphyrin levels are often elevated in the urine of AIP patients as well, with uroporphyrin predominant.[3] These porphyrins emit a fluorescent red colour when exposed to light. In addition, dypyrromethane, a degradation product of PBG as well as the cofactor of PBGD, is a brown colour.[160] Thus it may be a combination of porphyrins and dipyrromethane that results in the brownish-red 'Coca-Cola' colour of urine in AIP patients.[160]

Although initial diagnosis is through heme precursor levels in urine, diagnosis can also be confirmed by determination of PBGD activity levels in erythrocytes. PBGD activity is normally reduced in both acute and latent forms of AIP to about 50% of normal activity.[172] However, there is an overlap of activity levels between porphyria and normal patients so the disorder cannot always be exclusively diagnosed through determination of PBGD activity. DNA analysis is by far the most definitive diagnostic tool but with more than 100 reported mutations of PBGD in AIP it is also very complicated.[3, 161]

3.2.3 Hereditary Coproporphyria (HCP)

HCP combines the clinical features of the acute and cutaneous porphyrias. It presents with approximately 50% diminished activity of CPOX leading to overproduction of coproporphyrinogen which inhibits PBGD activity (Figure 2.1).[93] Acute symptoms are similar to AIP although this porphyria is less severe. Onset of acute symptoms usually occur after puberty which is characteristic of the acute porphyrias due to increased hormone levels. Levels of ALA exceed PBG, and HCP presents with elevated coproporphyrin levels in stool as well as mildly elevated levels in urine. ALA and PBG levels return to normal more easily between attacks than in AIP. Cutaneous symptoms manifest as vesculo-bullous dermatitis on sun-exposed skin; usually the face and hands. Skin lesions often heal with changes in pigmentation and scarring.

3.2.4 Variegate Porphyria (VP)

The enzyme affected by a genetic defect in VP is PPOX, which is present at approximately 50% of normal enzyme activity. This disorder is most highly prevalent in South Africa due to a founder affect with up to 3/1,000 of the population with the genetic defect. VP presents with increased levels of ALA, PBG, coproporphyrins and protoporphyrins. Protoporphyrin is the major accumulated and excreted porphyrin. As is the case in HCP, coproporphyrinogens and protoporphyrinogens that accumulate in VP inhibit PBGD activity[93] and, as a result, the acute symptoms of VP also mimic those seen in AIP. As with AIP and HCP, acute symptoms generally do not develop till after puberty. In addition to acute attacks, VP also presents with cutaneous symptoms similar to HCP although more common and severe. Photosensitivity and

dermatitis often develop at an early age with ulcers forming after minimal trauma to light exposed skin that often result in scarring.

Initial diagnosis is conventionally through fecal porphyrin levels, although diagnosis can also be through porphyrin levels in the urine and plasma. DNA analysis is the best diagnosis method as excretion of precursors are often normal between acute attacks, unlike AIP.

VP has been associated clinically with George III of Britain as well as related royalty and thus may have influenced world events although this claim is controversial[4, 175] and the royal family denies it.

3.3 The Cutaneous Hepatic Porphyrias

The hepatic porphyrias can be described as "acute" or "chronic". The acute hepatic porphyrias include ALAD-P, AIP, HCP, and VP (discussed above). The chronic hepatic porphyrias, or hepatic cutaneous porphyrias, do not present with acute neurovisceral symptoms and include PCT and HEP which are due to partial (~50%) or near total (>90%) deficiency of UROD respectively. PCT is the more mild of the two and occurs mainly in adult men with underlying liver disease and iron overload. Severe UROD deficiency results in HEP which manifests in childhood or infancy and continues lifelong with more severe skin symptoms then found in PCT.

3.3.1 Porphyria Cutanea Tarda (PCT)

PCT is the most common form of porphyria. There are three different types of PCT.[176] Type I (70-80% of cases) is sporadic without familial history and presents with normal erythrocyte UROD activity but decreased hepatic UROD activity. Type

II PCT (~20% of cases) is familial, inherited in an autosomal dominant pattern, and characterized by decreased UROD activity in both red cells and in the liver. Type III PCT (>5% of cases) is also familial, however, erythrocyte UROD activity is normal and this form is solely due to decreased UROD in hepatocytes. In addition to the inherited defect in type II and type III, additional acquired factors are still essential for development of the disorder and those with partial (heterozygous) hereditary defects often remain asymptomatic. Multiple factors are known to have an influence and effect on UROD activity in this disorder and include hepatis C infection, estrogen, human immunodeficiency virus (HIV), and increased hepatic iron content.

Chile has the highest frequency of familial PCT with 50% of patients having Type II PCT.[177] In comparison, a study of 152 PCT patients in Barcelona, Spain showed 25 with familial PCT (16.4%) while the remaining 127 PCT patients (83.6%) were classified as having sporadic PCT.[178]

UROD activity is decreased to 50% of normal enzyme activity in the different types of PCT. In the acquired form, enzyme activity is reduced in the liver alone and is potentially reversible. In the inherited form, type II, activity is reduced in peripheral red blood cells in addition to the liver and the enzymatic defect lasts lifelong. UROD catalyses the decarboxylation of both the isomer I and the isomer III series of uroporphyrinogens (Figure 2.8). UROD carries out decarboxylation of the isomer I series which is not metabolised beyond coproporphyrinogen I at a slower rate than the isomer III series which is a precursor to heme. Uroporphyrins and hepta-carboxylporphyrins accumulate in the liver and both of these porphyrins can be detected in the plasma, urine and stool (Table 3.1).

The main clinical features of PCT are acute photosensitivity, mechanical skin fragility, and liver damage. The hepatic abnormalities of this disease do not result in acute attacks. The cutaneous symptoms are a result of interactions between UV radiation from the sun and high amounts of circulating porphyrins which originate from the liver and accumulate in the skin.[179] The skin symptoms can manifest as blisters, bullae, vesicles, and sores that occur on UV light-exposed areas; most often the face, hands, forearms and legs. These symptoms are not the result of acute photosensitivity but are rather the result of mild trauma to sun-exposed skin. The fluid-filled vesicles that form rupture easily and often heal slowly, becoming crusted and resulting in scarring. The skin lesions of PCT are identical to those in the acute hepatic 'mixed' porphyrias HCP and VP. The liver disease resulting from this porphyria is often associated with alcohol abuse, alcoholic liver disease, iron loading, and cigarette smoking. A review of 132 PCT patients in Italy reports that 66% of these patients were alcohol abusers at the onset of PCT.[180] In the acquired form of this porphyria, alcohol is the major precipitating agent with excessive intake in more than 90% of acquired PCT patients.[181] Factors leading to oxidative stress in the liver are thought to increase oxidation of uroporphyrinogen either to its corresponding porphyrin or to other intermediates which inhibit UROD activity. Uroporphyrin levels are greater than coproporphyrin levels in PCT compared to levels found in HCP and VP.

3.3.2 Hepatoerythropoietic Porphyria (HEP)

HEP is an uncommon inherited cutaneous porphyria that is related to type II PCT and results in a very severe (>90%) deficiency of UROD in both erythrocytes as well as hepatocytes.[182] It presents in infancy with severe photosensitivity with the formation

of blisters and vesicles similar to those in CEP (Section 3.4.2). This porphyria presents with lifelong massive overproduction, accumulation, and urinary excretion of uroporphyrins and heptacarboxyl porphyrins (Table 3.1). Accumulation of zinc-protoporphyrin is also found in erythrocytes distinguishing it clinically from PCT and CEP. Because the defect of this porphyria occurs in all tissues, a bone marrow transplant or a liver transplants on their own is not curative.[183]

3.4 The Cutaneous Erythropoietic Porphyrias

The cutaneous erythropoietic porphyrias include CEP, EPP and the recently described XLPP. The cutaneous erythropoietic porphyrias are characterised by excess excretion of porphyrins rather than the heme precursors ALA and PBG. The predominant symptom is extreme photosensitivity with resulting skin damage. This can present as lesions with skin fragility, sub-epidermal bullae, hypertrichosis, hyper- and hypo-pigmentation. As mentioned previously, porphyrins absorb light at a wavelength of 400-410 nm, and interactions between high levels of circulating porphyrins and light result in porphyrin-induced photosensitivity.[179, 184] For control of skin symptoms, protective clothing and opaque sunscreens are recommended. Normal sunscreens are not beneficial as they do not screen out solar radiation of 400-410 nm.[185]

3.4.1 X-linked Protoporphyria (XLPP)

This is a rare porphyria due to a gain of function mutation of the erythroid-specific ALAS2 gene leading to over-expression of ALAS2 activity.[77-79, 79] The over-activity of ALAS2 in XLPP leads to an accumulation of protoporphyrin IX in erythrocytes as well as other tissues resulting in photosensitivity and liver disease. As there is more protoporphyrin production than heme requirement, FECH, the enzyme that converts

protoporphyrin IX into heme through the insertion of iron, uses zinc as an alternative substrate resulting in zinc-protoporphyrin accumulation in the erythrocytes.[79] The symptoms of this porphyria are indistinguishable from the cutaneous photosensitivity of EPP, the porphyria resulting from impaired FECH activity (Section 3.4.3).

3.4.2 Congenital Erythropoietic Porphyria (CEP)

CEP is a rare porphyria due to diminished activity of UROS.[186, 187] The diminished activity (<10%) of UROS results in accumulation of hydroxymethylbilane which is then non-enzymatically converted to uroporphyrin I. As uroporphyrin I plays no further role in the heme pathway (Figure 2.1), levels of uroporphyrin I as well as coproporphyrin I isomers are elevated. Thus this porphyria presents with gross overproduction of uroporphyrin I as well as other porphyrin I isomers. Porphyrins accumulate in the normoblasts of bone marrow and are excreted into the urine and feces. Porphyrins deposited in bone and teeth result in pink-brown discolouration, which fluoresces bright red at 400 nm.

This porphyria manifests from birth with photosensitivity and variable clinical presentations including formation of vesicles and bullae, which often results in facial scarring, hirsutism, and fluorescent teeth (erythrodontia).[3] Severe cases of recurrent vesicles and infection can lead to scarring and deformities including loss of digits and facial features including the eyelids, nose, and ears. Changes in bone structure are also often seen in CEP and are thought to be due to vitamin D deficiency as light avoidance is a necessity in this porphyria[188, 189] although bone changes may also be due to porphyrin deposits.[190]

3.4.3 Erythropoietic Protoporphyria (EPP)

EPP is more common than CEP and is a result of reduced FECH (15-25%) activity. This is also a chronic cutaneous porphyria and presents in early childhood with pain, burning, and swelling of skin with sun exposure as well as exposure to other UV lights. This usually occurs after only brief exposure and can develop within minutes.[191] With continuous exposure, erythema, edema, and itching become worse[191] and symptoms are described as an unbearable burning sensation of sun exposed skin. Burning and itching can occur without any skin damage. Vesicles are uncommon in this porphyria although the skin of patients with chronic, repeated sun exposure often becomes leathery. Increased skin fragility and deformed facial features and digits are not characteristic of this porphyria; teeth do not fluoresce and neurovisceral symptoms do not occur. Patients can, however, develop liver disease due to high levels of free protoporphyrins leading to organ damage.

Protoporphyrins are increased without high levels of coproporphyrins in the plasma, red cells, and sometimes stool of EPP patients. Urinary porphyrins are normal as protoporphyrins are not excreted in the urine. Lipid-soluble protoporphyrins deposited in dermal blood vessels are photo-activated with sun exposure leading to the characteristic skin changes seen in this porphyria. It is photo-activation of the water-soluble uroporphyrins, on the other hand, that lead to the chronic blistering seen in CEP and PCT.

3.5 Other Disorders Involving Heme Biosynthesis

In addition to the porphyrias discussed above, X-linked sideroblastic anemia, iron deficiency anemia, lead poisoning and tyrosemia are also the result of altered heme metabolism.

3.5.1 X-Linked Sideroblastic Anemia (XLSA)

XLSA is a result of reduced ALAS activity. This is a recessive disorder and results in iron accumulation due to lack of protoporphyrin IX synthesis.[75, 76, 192, 193] The excess iron in bone marrow is deposited in the erythroblast mitochondria clustered around nuclei as non-ferritin iron; hence the descriptive term 'ringed sideroblasts'. Iron overload resulting from increased iron delivery to the tissues eventually leads to secondary hemochromatosis which may result in diabetes, liver and heart failure without treatment.[194, 195] This disease is typically pyridoxine-responsive[114, 115] as pyridoxal-5-phosphate is an essential cofactor of ALAS and helps restore ALAS activity.

3.5.2 Iron deficiency anemia

Iron deficiency anemia, as its name suggests, is a result of iron deficiency usually from the diet. As iron is essential for the final step of heme formation, as iron deficiency results in a heme deficiency and reduced capacity to transport oxygen to and from tissues. Mild iron deficiency anemia is common in menstruating women and is often associated with vegetarian diets. In severe cases of iron deficiency, erythrocyte protoporphyrin elevations are elevated as FECH requires iron to complex with protoporphyrin to form heme (Figure 2.1). These elevated protoporphyrin levels rarely reach those seen in EPP and protoporphyrin is usually found complexed with zinc instead.[196]

3.5.3 Lead poisoning

Lead is an inhibitor of ALAD and the symptoms of lead poisoning resemble those of the acute porphyrias.[197] Like ALAD-P, ALA is elevated without PBG elevation. If treated early this acquired 'acute porphyria' is reversible. Removing exposure to lead

as well as lead removal from the body through chelating agents can help reverse the symptoms. It has been suggested that ancient Romans may have been exposed to high levels of lead in their tap water. If this is the case, this 'acquired porphyria' may have influenced world events.

3.5.4 Hepatorenal tyrosemia

Hepatorenal tyrosemia is a result of deficiency of fumarylacetoacetate hydrolase, an enzyme of the degradation pathway of the amino acid tyrosine. Deficiency of this enzyme results in a buildup of succinylacetone which has an inhibitory effect on ALAD.[198] Inhibition of this enzyme can result in an altered heme metabolism similar to that of ALAD-P and lead poisoning.[56]

3.6 Summary of Chapter 3

Disruption of heme biosynthesis can result in a variety of disorders of metabolism, mainly the porphyrias. Porphyria, in severe cases, can present with poor quality of life and occasionally fatalities. The incidence of the porphyrias may be underestimated,[7, 199] which can be dangerous. If unrecognized or misdiagnosed, a porphyria patient may be prescribed porphyrinogenic drugs which will only aggravate symptoms and can potentially be life-threatening. Further investigations into the heme biosynthetic pathway will result in a better understanding of these disorders and their complex biochemistry.

CHAPTER 4. TREATMENT OF THE PORPHYRIAS

4.1 The Search for a Prophylaxis – Background

The search for safe and effective treatment of the porphyrias is an ongoing area of research. Successful treatments provide insight into the biochemistry and the pathophysiology of the porphyrias; the amelioration of the disordered biochemistry gives clues as to what is disordered in the underlying biochemistry. As the porphyrias are genetic disorders resulting from mutations of enzymes, treatment and control of symptoms are more likely to be established than a cure. Most patients with a mutation of an enzyme in the heme biosynthetic pathway remain asymptomatic with a slight increase in heme precursor levels and normal levels of heme. Many drugs and chemicals as well as fasting, hormones, stress and exercise are known triggers of acute porphyric attacks. Tolerance of some triggering factors may vary from porphyria to porphyria and sometimes from patient to patient. The most commonly recommended and important method for control of porphyria symptoms is avoidance of the triggering factors that are known to precipitate symptoms. This requires early diagnosis and education of both doctors and patients regarding porphyria and its triggers, especially in relation to drugs that induce cytochromes P450 and thus precipitate acute attacks. There are currently many readily available safe and unsafe drug lists for use in the porphyrias.[200]

Avoidance of triggering factors is the first line of defense in the acute hepatic porphyrias. However, treatment is required once a patient is in an acute attack as the symptoms can lead to respiratory paralysis and death. The major target of treatment of the acute porphyrias is repression of ALAS1, the rate-limiting enzyme of the hepatic heme biosynthetic pathway, and thus reduction of ALA and PBG accumulation, see

Figure 2.1. Traditionally the acute attack has been and is treated with glucose which represses ALAS1 activity[165] although heme arginate now appears to be the preferred method of treatment which also represses ALAS1 activity through feedback inhibition (Figure 2.1).[201] Severe cases of acute porphyrias have been treated with liver transplants which in most acute porphyria cases reverses the porphyria[202-207] highlighting the connection of these porphyrias to the liver. Alternatively there is a long history of the porphyrias being treated with micronutrients, especially B vitamins (Table 4.3). However, this method of treatment is geographically localized and does not appear to be popular in the current literature and is rarely mentioned in current reviews. In Argentina, the acute porphyrias have been successfully treated with folate in addition to vitamin B-complex and glucose syrup for over 30 years,[24] yet papers recording this are published in Spanish and thus are not readily available to the English-speaking world. As discussed in Section 2.3, micronutrients play a major role in heme synthesis (Table 2.1). Is dismissal of porphyria treatment with micronutrients in the current literature justified? This chapter will review reports of micronutrient therapy as well as the other previously discussed treatments of porphyria.

4.2 Treatment with Glucose

One of the recommended approaches for treatment and prevention of acute porphyria attacks is a high carbohydrate diet or intravenous glucose therapy. Acute attacks can be triggered through fasting or low calorie diets[165] and, prior to heme arginate availability (Section 4.3), carbohydrate loading was the standard treatment.[201] The beneficial effect on the porphyrias attributed to carbohydrates has been termed the 'glucose effect'.[165] The underlying mechanism of the 'glucose effect' is through suppression of ALAS1 expression by glucose. This slows down the overproduction of

the precursors ALA and PBG and has been reported to alleviate symptoms.[165, 208-211] Glucose does not, however, correct the altered heme biosynthesis in the acute porphyrias as it is deficient activity of enzyme(s) further down the pathway that result in the majority of these disorders. Effects of glucose therapy are not consistent and vary from improvement to no improvement.[212]

Increased carbohydrate intake through the diet including glucose-containing drinks and high energy foods are beneficial only in prophylaxis of mild attacks. Here we will focus mainly on intravenous glucose therapy.

4.2.1 Glucose Therapy

Glucose is the primary metabolic energy source in humans and plays a key role in ATP and NADPH generation via glycolysis and the TCA cycle, thus maintaining positive energy balance in the cell which is important for healthy cell growth and function. Blood glucose levels are maintained by the balance between glucose uptake by peripheral tissue and glucose secretion by the liver. The hormone insulin facilitates uptake of glucose from the blood into the cells where it is utilised for essential growth and energy requirements. When mammals fast, glucose homeostasis is achieved by triggering expression of gluconeogenic genes in response to glucagon and glucocorticoids. The pathways act synergistically to induce gluconeogenesis (glucose synthesis). Insulin controls hepatic glucose output by counteracting the actions of glucagon and catecholamines in the liver. In summary, glucagon raises the blood glucose during fasting whereas insulin lowers blood glucose during feeding.

As mentioned before, the beneficial effect glucose has on the porphyrias appears to be due to repression of ALAS activity,[208] although the mechanism by which this occurs

is not fully understood. Regulation of ALAS expression during fasting and feeding also appears to be mediated by the counter-regulatory hormones insulin and glucagon.[213, 214] Insulin has been found to indirectly inhibit ALAS gene expression[213, 215, 216] with insulin treatment of primary mouse hepatocytes resulting in reduced basal levels of ALAS mRNA.[74] Insulin and glucose together have a greater effect on reducing ALAS expression than insulin alone suggesting that glucose also plays a role in ALAS transcription.[74] It is possible that the glucose effect may be a result of increased insulin levels in response to glucose rather than glucose itself.

The effect of glucose and insulin on ALAS may be explained at the transcriptional level through PGC-1. PGC-1 is a coactivator of nuclear receptors and other transcription factors that control mitochondrial biogenesis and oxidative metabolism in many tissues.[217, 218] PGC-1 also appear to play a role in the control of ALAS expression in the fasted and fed liver at the transcriptional level.[74, 219] *In vivo,* PGC-1 is induced in the liver during fasting and stimulates expression of the ALAS gene in hepatocytes.[74] These elevated PGC-1 levels have been shown to cause acute attacks in induced porphyrias in rats.[74] Because of this, drugs that elevate hepatic PGC-1 levels should be avoided in patients with hepatic porphyrias. High levels of insulin appear to down-regulate the expression of PGC-1.[219] Glucagon induces PGC-1 expression, and rising blood glucose reduces glucagon secretion.[218, 219] This suggests that PGC-1 may be the major target of the "glucose effect" in the porphyrias.[218] If PGC-1 expression is reduced by insulin and PGC-1 induces ALAS expression, the lack of induction of ALAS expression by PGC-1 in the presence of insulin may be the mechanism by which glucose and the subsequent elevation of insulin can ameliorate an acute porphyric attack.[74]

4.2.2 Complications of Glucose Therapy

Although glucose has been recommended as a porphyria treatment, there are potential complications that can result from intravenous glucose therapy or high carbohydrate diets which include weight gain and increased risk of developing diabetes mellitus.

4.2.2.1 Diabetes and Weight Gain

Diabetes mellitus is a metabolic disorder in which the patient has an inadequate insulin response, resulting in a propensity to hyperglycemia. Because high carbohydrate diets and intravenous glucose therapy offer relief from porphyric attacks, it is not unreasonable to suggest that diabetes-induced hyperglycemia could have a similar affect. There are reports of AIP porphyria patients who obtain better control and in some cases cessation of symptoms after developing diabetes.[220-222] However, a case report suggests that diabetes can also antedate AIP with porphyria symptoms developing in a patient 12 years after hyperglycemia which was controlled through herbal medications.[223] It is worth noting that in this case there were fewer acute attacks as the patients glycemic control diminished.[223]

Interestingly, it has also been reported that diabetes is prevalent in patients with the cutaneous hepatic porphyria PCT.[178, 224, 225] A study of 81 PCT patients showed 41% (33 patients, 25 with diabetes mellitus and 8 with impaired fasting glucose) having glucose metabolism alterations.[224] A previous study by the same authors of 152 patients showed a 23.7% prevalence of diabetes mellitus and 11.9% prevalence of impaired fasting glucose.[178] This is in comparison to a 13% prevalence in the general population of the area.[226] As diabetes is highly prevalent in PCT, it is possible that alterations of porphyrin metabolism may correlate with insulin resistance.[225] It

appears that diabetes mellitus development results in symptomatic relief in patients with severe AIP. For those with latent AIP or PCT, diabetes may simply result in undesired diabetic complications.

4.2.2.2 Hyponatremia

Perhaps one of the most severe potential complications of intravenous glucose is the risk of hyponatremia. Stein et al. [227] reports a tragic case of a 17 year old school girl presenting with AIP suffering from a severe acute attack. She was treated with 50% glucose infused through a central venous line and suffered from respiratory arrest. Post mortem suggested this was due to profound hyponatraemia aggravated by intravenous glucose solution.[227] Glucose and Na^+ levels have to be carefully balanced as serum Na^+ concentration decreases with increasing glucose concentration as water shifts from the intracellular to extracellular compartment.[228] Hyponatremia is a common complication in AIP,[229-233] making intravenous glucose a potentially dangerous option.

4.2.2.3 Micronutrient Depletion

Depletion of micronutrients is also a concern in glucose therapy. A study of nine healthy patients who ingested a glucose solution after a 12 hour fast resulted in a decrease in plasma concentration of PLP.[234] PLP is the active form of vitamin B6 and as already mentioned is an important co-factor in over 300 enzymes including the ALAS catalyzed reaction in the heme biosynthetic pathway. Due to the increased activity of the PLP-dependent, rate-limiting enzyme ALAS in the porphyrias, PLP may already be depleted (Section 4.5). Thus, intravenous glucose may exacerbate a pre-existing vitamin B6 (PLP) deficiency in porphyria patients. Another potential

complication of intravenous glucose therapy is thiamine deficiency resulting in Wernicke's encephalopathy.[235] Wernicke's encephalopathy is a life threatening neurological disorder that results from thiamine deficiency.[235, 236] If any patient is treated with glucose infusions without having thiamine levels tested first, they run the risk of developing severe thiamine deficiency leading to Wernicke's encephalopathy.[237, 238] Severe thiamine deficiency leads to respiratory failure if untreated; respiratory failure is the main cause of death in an acute attack. Because of this, determining vitamin status should be a common procedure before glucose therapy in all patients requiring glucose infusions.

4.2.2.4 Summary of Glucose Therapy

The possible complications of glucose therapy include weight gain, diabetes, micronutrient deficiency, and hyponatremia as well as B-vitamin depletion. These potential side-effects of glucose therapy make it clear why glucose therapy is no longer the method of choice for treatment of the acute porphyrias.

4.3 Heme Therapy

4.3.1 Heme Treatment

Heme therapy involves the intravenous infusion of heme in the form of hemin or heme arginate (Figure 4.1); it will be assumed that the term 'heme' refers to either of these compounds. This therapy is well studied and widely used and is considered a safe method of treatment.[239] As heme regulates its own biosynthetic pathway through feedback inhibition of ALAS1 (Figure 2.1), 'heme' infusions lower ALAS1, which reduces precursor accumulation, and results in symptom relief.[230, 240] 'Heme infusions

also supply the body with heme which may be deficient due to the enzyme 'block' in heme synthesis in porphyria.

Hemin (trade name Panhemin) consists of protoporphyrin IX with an iron ion and a chloride ligand, whereas heme arginate (trade name Normosang) is a compound of heme where arginine is added to prevent rapid degradation (Figure 4.1). A study suggests that heme arginate is more stable than hemin.[241] However, both forms are widely used. Normosang appears to be the heme of choice in Europe, whereas Panhemin is mainly used in the United States.[201] These heme products are the most popular forms for use in heme therapy with recent studies showing successful symptom control upon administration.[230, 242-244]

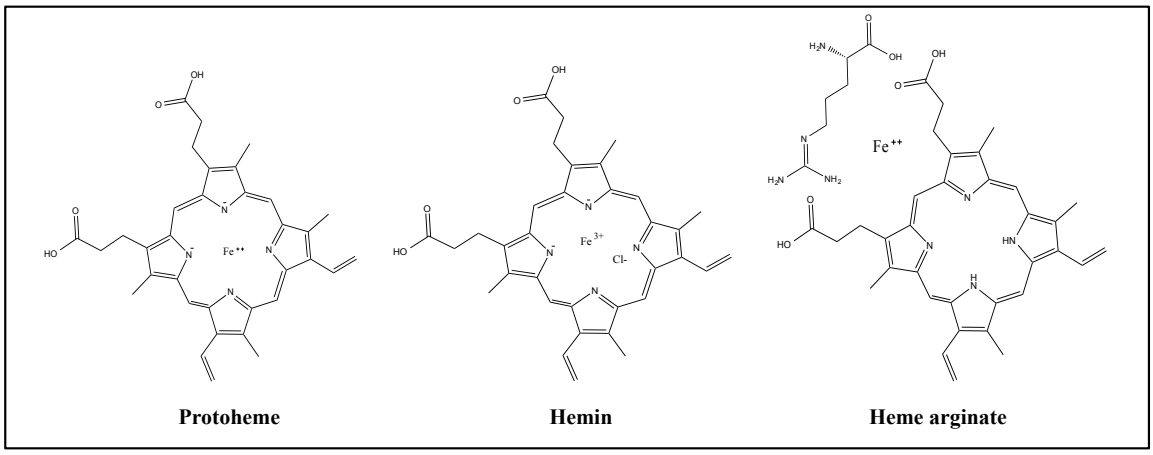

Figure 4.1 Structures of Heme.

The structure of protoheme is compared to that of hemin and heme arginate, the commonly used forms of heme for porphyria treatment.

4.3.2 Complications of Heme Therapy

Heme therapy improves symptom control, but does not appear to reverse any established neuropathy although it may prevent further neuronal damage.[201] Potential complications of frequent 'heme' infusions are hemochromatosis, hypotension,[245]

coagulopathy,[246] acute renal tubular damage, and phlebitis.[202] In addition to these potential side-effects, 'heme' therapy is a very expensive treatment with a standard four-day treatment costing approximately $8,000 US,[247] and is not readily available in some countries, for example, India.[245]

Frei et al.[203] reports a case of an AIP patient who received an accidental 6-fold overdose of heme arginate that resulted in severe coagulation and acute liver failure resulting in an emergency liver transplant. Although the patient was doing well a year after the successful liver transplant, liver transplantation may have been averted if a normal dose had been given. Medical error is thus another potential complication of heme therapy.

4.4 Liver Transplantation.

4.4.1 Treatment with Liver Transplantation

Liver transplantation has successfully reversed acute porphyria[202-207] resulting in normalisation of heme precursors. In addition, porphyric livers from AIP patients transplanted into non-porphyric patients have resulted in AIP developing in these patients.[248, 249] These cases highlight the importance of the liver in the pathophysiology of this disorder. Liver transplantation imports a pristine hepatic biochemistry into the patient and reverses the porphyria. This is not a permanent treatment for the acute porphyria ALAD-P however,[169] suggesting that in this porphyria more than the liver may be affected.

Liver transplants have also been reported to successfully treat liver disease in EPP patients[250-252] although a review reports EPP reoccurrence in 11 out of 17 EPP

patients with liver transplants[253] as EPP is an erythropoietic rather than hepatic disorder.

4.4.2 Complications of Liver Transplants

Dowman et al.[254] who reviewed liver transplantation in ten AIP patients reported an 80% success rate and a high rate of hepatic artery thrombosis. They also observed that previous neuronal damage is not improved by transplantation. The two patients in this study who died did so from multi-organ failure. The other eight were free of porphyria symptoms after surgery. In addition, anesthetics, as mentioned earlier, can cause an acute attack[255] and anesthetics must be selected carefully before any surgery is performed on an acute hepatic porphyria.

As porphyrins are excited by UV light resulting in photo-cutaneous and photo-visceral damage, special filters are needed on all lighting if surgery is performed on a porphyria patient presenting with cutaneous symptoms and porphyrin accumulation.[253, 256] Porphyrin accumulation in the intra-abdominal tissue can result in severe damage to this tissue and surrounding organs if exposed to UV light. However, Wahlin et al.[256] reported two patients with phototoxic injury even though they were protected by filters during surgery.

Although liver transplantation appears to correct the acute porphyrias in most cases, it is not always successful[254, 255] and factors that could alter the outcome include choice of anesthetics as well as appropriate light filters if cutaneous symptoms are present (VP and HCP in addition to the cutaneous porphyrias).

4.5 Micronutrient Therapy

It has been made clear that nutrients play a significant role in the heme biosynthetic pathway and the nutrients required range from macronutrients such as glucose (Section 4.2) to the micronutrient cofactors essential for enzyme function (Table 2.1). Atamna et al[58, 257] have suggested that inadequate dietary intake of micronutrients results in altered heme biosynthesis, which leads to mitochondrial decay and oxidative damage to DNA, and that this could be a factor in the ageing process. Inadequate amounts of dietary micronutrients also lead to a decline in critical enzymatic activities of the electron transport complexes. This increases the production of reactive oxidants and the functional decay of mitochondria.[257, 257] Could micronutrient deficiencies, if they have such an effect on heme synthesis also play a role in the porphyrias? Could the increased enzymatic activity of the porphyrias prior to the 'bottleneck' in the pathway deplete micronutrient cofactors resulting in micronutrient deficiencies?

Ames et al.[258] present evidence supporting the treatment of genetic disorders resulting from defective enzymes with high-dose vitamin therapy of the vitamin component of the corresponding coenzyme. In his discussion, Ames uses vitamin B6 (pyridoxal) treatment of sideroblastic anemia to boost ALAS activity as an example. He also suggests that VP may be treated with riboflavin. This treatment may partially restore the deficient enzyme activity and increase the enzyme's affinity for the corresponding cofactor thus relieving some of the symptoms.[258]

4.5.1 Micronutrient Deficiencies in Porphyria

Many of the symptoms of vitamin deficiencies are similar to those presented by the porphyrias (Table 4.1). While these possible connections may be unrelated, it is possible that the correlations between vitamin deficiency symptoms and symptoms of the porphyrias could provide a new perspective and understanding of the pathophysiology of the porphyrias. Consumptive tissue depletion of essential micronutrients during acute porphyria attacks could result from the enhanced condition of the heme pathway caused by abnormally high enzyme activity prior to the enzyme 'bottleneck' characteristic of the porphyria.

Vitamin deficiencies are common in the general population.[257, 259] Consumptive tissue depletion of vitamin cofactors during an acute attack could thus aggravate underlying deficiencies. Due to the many inter-relationships between levels of different nutrients, depletion of one or more nutrients can result in the depletion of others resulting in a cascading effect (Section 2.3). This, along with overproduction of precursors, would affect various pathways generating a variety of symptoms depending on where the metabolic imbalance resides in a particular patient. Table 4.1 reports some commonly reported porphyria symptoms along with analogous symptoms resulting from vitamin deficiencies.

Garcia-Diz did a dietary study of 16 AIP patients through a lifestyle questionnaire which showed an increased risk of malnutrition.[260] Porphyrias have been treated with micronutrients for over 60 years.[261] although this is not considered a mainstream treatment in the medical literature written in the English language. Different micronutrients can support the heme biosynthetic pathway through essential and non-essential co-factor roles as well as protect the body against oxidative stress and free

Table 4.1 Systems of Porphyria Similar to Those in Micronutrient Deficiencies.

Porphyria symptoms	Micronutrient deficiencies with analogous symptoms
Peripheral Neuropathy (acute)	Thiamine, Vitamin B6, Vitamin B12, Vitamin E
Severe abdominal pain (acute)	Calcium, Folate, Pantothenate, Vitamin B12, Vitamin D
Constipation (acute)	Niacin, Thiamine, Vitamin B12,
Nausea, vomiting (acute)	Folate, Magnesium, Vitamin B12, Vitamin D
Diarrhea (acute)	Folate, Niacin, Vitamin B12, Zinc
Liver problems	Vitamin B6, Vitamin D
Back pain (acute)	Calcium, Vitamin D
Headaches (acute)	Magnesium, Vitamin B12, Vitamin D
Pain in limbs (acute)	Thiamine, Vitamin B6, Vitamin B12, Vitamin D
Edema	Niacin, Thiamine, Vitamin B6, Vitamin B12
Dysthesias and parasthesias (acute)	Biotin, Pantothenate, Vitamin B12
Muscle weakness (acute)	Copper, Folate, Magnesium, Niacin, Pantothenate, Thiamine, Vitamin B12, Vitamin D, Vitamin E
Muscle cramps (acute)	Biotin, Calcium, Pantothenate, Thiamine
Muscle paralysis (acute)	Vitamin B12
Seizures (acute)	Magnesium, Vitamin B6, Vitamin B12, Vitamin D
Tremors (acute)	Magnesium, Riboflavin, Thiamine, Vitamin B6, Vitamin B12, Vitamin E
Depressed or absent tendon reflexes (acute)	Magnesium, Thiamine, Vitamin B12
Difficulty breathing (acute)	Copper, Vitamin B12, Vitamin D
Arrhythmias (acute)	Copper, Folate, Magnesium, Thiamine, Vitamin B12
Hypertension (acute)	Lipoic Acid, Magnesium, Vitamin D
Lowered immunity (acute)	Calcium, Copper, Lipoic acid, Vitamin A, Vitamin C
Fatigue (acute)	Copper, Folate, Magnesium, Pantothenate, Vitamin B6, Vitamin B12, Vitamin D, Vitamin E
Depression (acute)	Biotin, Folate, Magnesium, Thiamine, Vitamin B6, Vitamin D, Vitamin C, Vitamin B12
Psychosis, paranoia, hallucinations (acute)	Vitamin B12
Irritability (acute)	Folate, Magnesium, Thiamine, Vitamin B12, Vitamin D
Delirium (acute)	Magnesium, Vitamin B12
Confusion (acute)	Folate, Niacin, Vitamin B12, Vitamin D
Insomnia (acute)	Calcium, Folate , Magnesium, Niacin, Pantothenate, Thiamine, Zinc
Anxiety/Restlessness (acute)	Magnesium, Pantothenate, Vitamin D
Anemia	Biotin, Copper, Folate,, Riboflavin, Vitamin B12, Vitamin E, Zinc
Skin photosensitivity (cutaneous)	Riboflavin

Porphyria symptoms	Micronutrient deficiencies with analogous symptoms
Itches (cutaneous)	Vitamin B6, Vitamin C
Blisters (cutaneous, not found in EPP)	Vitamin B6, Vitamin E
Rashes (cutaneous)	Biotin, Vitamin B6
Changes in skin pigmentaton (cutaneous)	Copper, Folate, Vitamin B12
Slowed wound healing (PCT)	Niacin, Vitamin C, Vitamin E, Zinc
Nail abnormalities (EPP)	Calcium, Zinc,
Skin lesions (cutaneous)	Niacin, Zinc

Table 4.2 Reports of Micronutrient Deficiencies Recorded in Porphyria.

Micronutrient deficiency	Porphyria	Number of patients	Reference
Riboflavin	HCP	1	[8]
Vitamin B6	AIP; VP	11; 1	[9]
	AIP	4	[10]
	AIP	13/24	[11]
	AIP	2	[12]
	AIP	21	[13]
	AIP	1	[14]
Folate	AIP	1	[15]
Vitamin A	PCT	34	[16]
Vitamin E	PCT	34	[16]
Vitamin C	PCT	7	[17]
	PCT	13	[18]
Vitamin D	HCP	1	[8]
	EPP	22/48	[19]
	EPP	84% of 42	[20]
	EPP	126/201	[21]
Magnesium	Acute attacks	“Common”	[22]

Cases of porphyria patients presenting with micronutrient deficiencies.

radicals through antioxidant properties. There have been many micronutrient deficiencies reported in the porphyrias (Table 4.2); here we will provide a much needed review on reports of micronutrient therapy of the different porphyrias.

4.5.2 Treatment of the Porphyrias with Micronutrients

Over the past sixty years there have been numerous reports of successful treatments of the acute and cutaneous porphyrias with a variety of micronutrients (Table 4.3). However, vitamin therapy is rarely included in reviews of porphyria treatments.[3, 201, 247, 262, 263] and if it is, it is usually dismissed. Is this dismissal justified? Could micronutrient therapy be a safer alternative to heme arginate treatment? As mentioned in Section 4.1, folate in addition to a vitamin B-complex is reported as the main treatment and preventative for acute attacks in Argentina.[24] Reports of successful treatment of AIP with folate (30 mg), vitamin B-complex (vitamin B1, 15 mg; vitamin B2, 15 mg; vitamin B6, 5 mg; vitamin B12, 5 mg; vitamin B5, 20 mg; vitamin B3, 100 mg; vitamin C, 600 mg; vitamin E, 30 U) and two to four tablespoons of glucose syrup per day has been reported to have already successfully treated over 65 patients in 1985;[24] this is still the main method of treatment in Argentina today. Reported micronutrient treatment of each of the major classes of porphyria is presented below.

Table 4.3 Reported Successful Treatment of Porphyria with Micronutrients.

Porphyria	Micronutrient	Number of Patients	Amount	Reference
AIP	Vitamin B6 and glucose	1	---	23
	Vitamin B6 (pyridoxine)	4 in remission	40-60 mg/day	10
	Vitamin B6 (pyridoxine)	2	400 mg/day	12
	Vitamin B6 (pyridoxal)	2	100 mg, single dose	13
	Folate, B-complex, and glucose syrup	> 65	30 mg/day	24

Porphyria	Micronutrient	Number of Patients	Amount	Reference
	Folic acid	3	30 mg/day	272
	Folate and red blood cell infusions	1	0.5 mg/day	15
	Zinc	1	220 mg/8hours	27
	Magnesium Sulfate	1	3 g then 1 g/hour IV	28
VP	Vitamins C and E	12	---	29
	Vitamin E	2	---	30, 31
HCP	Riboflavin	1	"large doses"	8
PCT	Pyridoxal-5-phosphate	4	500 mg/day IV	32
	Vitamin B6	2	500 mg/day IV	33
	Pyridoxal-5-phosphate	3	500 mg/day IV	34
	Riboflavin, niacin, and Vitamin C	39	---	37
	Thiamine, riboflavin, Niacin and Vitamin C	80	---	38
	Vitamin E	5	1 g/day	39
	Vitamin E	2	100 mg/day	41
	Vitamin E	7	1600 IU/day	31
	Vitamin E	5	---	43
	Vitamin E		---	44
	Vitamin B12 and folate	1	---	45
EPP	PLP	2	1 g/day and 300mg/day	46
	Vitamin C	8/12	1 g/day	47
	Beta carotene	7	50-250 mg/day	48
	Beta carotene	19/23	---	279
	Zinc	1	600 mg/day	50
	Cysteine	9/10	500 mg/twice daily	51
	N-acetylcysteine	1	400 mg/twice daily	53
	N-acetylcysteine	1	1200 mg/day	54
CEP	Pyridoxal-5-phosphate	1	30 mg/day subcutaneous injection	55

Reports of successful treatment of the different porphyrias with micronutrients. IV, intravenous; IU, international unit.

4.5.2.1 Treatment of the Acute Porphyrias With Micronutrients

(i) B-vitamins

As PLP (vitamin B6) is an essential cofactor for the production of neurotransmitters, vitamin B6 has also been used as a therapeutic adjunct in seizures, Parkinson's disease, depression, chronic pain, headache, behavioral abnormalities, peripheral neuropathies, carpal tunnel syndrome, and premenstrual syndrome.[120] As many porphyria symptoms are similar to those of B6 deficiency (Table 3.1), and in light of the therapeutic potential of vitamin B6 in other disorders, there is the potential for its use as a therapeutic aid in the porphyrias as well.[264] Treatments of the acute porphyrias with vitamin B6 stems from the hypothesis that there is consumptive tissue depletion of this vitamin due to excessive demand of the PLP-dependent ALAS in the porphyria state resulting in a vitamin B6 deficiency;[265] this will be discussed further in Section 5.4.

There have been case reports of AIP symptoms resolved following administration of vitamin B6 plus glucose,[23, 266] and recently vitamin B6 has been used to support AIP patients in remission.[9, 10] An earlier report suggests no clinical improvement in two AIP patients who were vitamin B6 (pyridoxine) deficient.[13] The two patients were given a single 100 mg dose of pyridoxine, however this may not have been enough to show any improvement whereas another study at a similar time reports progressive improvement in two severe cases over three months with vitamin B6 (PLP) 400 mg/day.[12] Since the acute symptoms of VP and HCP result from the depression of PBGD by protoporphyrinogen and coproporphyrinogen, treatments that are successful for AIP may also be effective for these two porphyrias.

Some livers are not capable of converting pyridoxine hydrochloride, the vitamin B6 form that is common in supplements, into PLP (the active coenzyme form of vitamin B6).[267] This is the case when folate, vitamin B2, and vitamin B12 levels are not sufficient.[268, 269] It is possible that this is also the case in the acute hepatic porphyrias. If so, supplementation with pyridoxine would not be effective and the PLP form of the vitamin should be administered. PLP has a half-life of 23.1 hrs in the circulation, and this may underpin the ineffectiveness of a single dose.[270]

The successful treatment of the acute porphyrias with folate originates from a report that folate administration enhances PBGD,[271] the enzyme deficient in AIP, thus normalizing the pathway and ameliorating the porphyria crisis.[15, 24, 272] This provides evidence in support of the cofactor treatment of metabolic disorders discussed earlier.[258] Folate treatment also normalizes acute symptoms in VP and HCP;[273] these porphyrias produce elevated levels of coproporphyrinogens and protoporphyrinogens which suppress PBGD activity through feedback inhibition (Figure 2.1) resulting in AIP-like symptoms.[93] Folate, B6, and B12 supplementation together have better results in treating a folate deficiency than folate alone suggesting that the B-vitamins should be given together to avoid depletion of any not given.[274] Folate (30mg) in addition to a commercial B-complex tablet and two to four tablespoons of glucose syrup daily has been reported to be beneficial in the control and suppression of acute attacks and is the treatment of choice in Argentina.[24, 275]

(ii) Antioxidants and Minerals

The antioxidants vitamin E and vitamin C have also been reported to successfully treat VP through decreasing plasma and neutrophil oxidative damage and restoring

PPOX expression.[29] Another report relating to two AIP patients observed no beneficial effects of these or other antioxidants.[276] If the antioxidants that successfully treated VP did so through restoring PPOX expression, this may explain why this treatment was successful in VP and not in AIP. One must also remember that due to the rarity of porphyria, it is difficult to get statistically significant results.

Melatonin is another important and powerful antioxidant which will be discussed further in Section 5.3.2. Melatonin has been shown to protect against ALA-induced oxidative damage, preventing cell injury.[130, 132, 277, 278] This suggests that melatonin may have potential in the treatment of the acute porphyrias.

In addition to B vitamins and antioxidants in treatment of the acute porphyrias, zinc sulphate has been reported to provide pain relief in AIP patients.[26, 27] The mechanism of this is suggested to be due to the remediation of a free zinc deficiency, resulting from zinc chelation by overproduced porphyrins. Some acute porphyria symptoms are similar to those in zinc deficiency. Magnesium has also been used as a therapeutic aid in acute attacks for the control of seizures.[28]

4.5.2.2 Treatment of Chronic Hepatic Porphyrias with Micronutrients

(i) B-vitamins

The non-acute porphyrias have also been treated with vitamins. Intravenous injection of PLP (500 mg/day) in four PCT patients decreased urinary porphyrin excretion and reduced serum iron levels.[32, 280] If PLP is a non-essential activator of ferrochelatase, the enzyme that inserts iron into heme, it could be playing a role here.[96, 97] A supporting study of two PCT patients treated by the same method (intravenous vitamin B6 500 mg/day for a month) resulted in relief of symptoms, including

complete disappearance of the erupting bullae and fragile skin.[33] There have been reports of B vitamins treating PCT in different countries and languages including German,[34, 36, 266] Russian,[37, 38] Croatian, [35] and Spanish [281].

(ii) Antioxidants

Antioxidants have treated the cutaneous hepatic porphyrias as well, with vitamin E as the main antioxidant used to treat PCT.[31, 40-43, 282] Vitamin E is an antioxidant and has been shown to reduce oxidative stress in PCT.[44] In addition, it is suggested that it increases UROD activity and thus lowers urinary excretion of porphyrins.[39] Vitamin E deficiency has been reported to decrease ALAD activity[40] and capacity to synthesise heme resulting in decreased levels of heme proteins;[283-286] this supports the hypothesis that it plays a role in heme synthesis.

4.5.2.3 Treatment of Cutaneous Porphyrias with Micronutrients

(i) B-vitamins

The cutaneous erythropoietic porphyrias have also been successfully treated with vitamin B6 leading to symptom relief and light tolerance in EPP.[46] This protective effect could be due to PLP's possible role in activating FECH,[96, 97] thus correcting its deficient activity.[96, 97] In a case of CEP, subcutaneous injection of pyridoxal-5-phosphate at 30 mg/day resulted in urinary porphyrin excretion returning to normal and cutaneous lesions disappearing within a year.[55]

(ii) Antioxidants and Minerals

Beta-carotene has also been reported to reduce photosensitivity and increased the time EPP patients could spend in the sun;[48, 279, 287, 288] vitamin C has been reported to provide possible benefits as well.[47] In addition, the antioxidant N-acetyl-cysteine has

been suggested to reduce photosensitivity in EPP.51, 52, 289 An emergency liver transplantation was averted in a case of EPP through intravenous N-acetyl-cysteine treatment; this case was continuously controlled successfully through ongoing N-acetyl-cysteine supplementation.[54]

Zinc sulphate has been reported to reduce photosensitivity in EPP likely through chelating free protoporphyrins.[50] Zinc-protoporphryins are less phototoxic than metal-free protoporphyrins.

4.6 Discussion

Heme biosynthesis is a complex pathway involving many factors and levels of control. It is tightly regulated and requires many nutrient cofactors that are either directly involved in the pathway or indirectly involved through related pathways that feed into the heme biosynthetic pathway. Heme and glucose both regulate the first step of the pathway, and although treatment of acute porphyria with heme and glucose has had positive results, there is a continuing need to investigate possible alternative treatments that may have similar results without some of the deleterious side effects such as weight gain[290] or diabetes.

Micronutrient depletions or deficiencies could play a role in the manifestation of some of the symptoms of porphyria because of their interplay with heme biosynthesis (Table 2.1). Interestingly, the peripheral neuropathy of vitamin B6 deficiency is very similar to the peripheral neuropathy of acute porphyria[120, 121] suggesting that PLP depletion may play a role.[12, 264] This is reinforced by the knowledge that vitamin B6 deficiency has been documented in the porphyrias. In Argentina, folate treatment of AIP is widely practiced and is known to aid and alleviate symptoms yet is more

effective when given with a vitamin B-complex as well.[24] These possible connections provide an area for further research.

If a nutrient cofactor is depleted through an overactive heme pathway, it may not be possible to obtain enough of the nutrient from the diet. Vegetarian, vegan, and Atkins diets, as well as diets high in processed food can result in nutritional deficiencies of certain nutrients further aggravating any underlying deficiency or deficiencies. Micronutrient deficiencies are prevalent in the general population with high rates of modest, non-symptomatic nutrient deficiencies.[291, 292] A porphyria patient with vitamin deficiencies may be more susceptible to an external provoking factor thus becoming symptomatic. Once a porphyric attack is triggered, nutritional deficiencies may become exacerbated as enzyme activity is up-regulated prior to the enzymatic 'bottleneck' in the heme biosynthetic pathway resulting in increased micronutrient cofactor consumption. If more of a particular nutrient is required than is possible to get from a normal diet, supplementation may be a possible therapeutic aid for relief of symptoms. Prevention of acute porphyric attacks by avoidance of triggering factors and by maintaining an optimal nutritional status is a safe approach toward prophylaxis of the porphyrias. Ames and his team[258] presented evidence in 2002 supporting the treatment of genetic disorders that are due to defective enzymes with high doses of the vitamin cofactor component of the corresponding enzyme. This may partially restore enzyme activity and increase the enzymes affinity for the corresponding cofactor, so relieving some of the symptoms in the porphyrias.

Another area of porphyria treatment being investigated is gene therapy. Recent pre-clinical studies of liver-directed RNA interference therapy for ALAS1 mRNA is a current area of research.[293] A separate study in macaque monkeys *Macaca*

fascicularis suggests intravenous infusion of PBGD into AIP patients could also correct the disorder by correcting the enzyme deficiency.[294] Eight AIP patients are currently in phase I trials of this therapy.[294]

In conclusion, micronutrient therapy appears to stand out as a safe alternative to heme and glucose therapy in the acute porphyrias. Even liver transplantation due to liver failure caused by EPP has been simply averted with high doses of N-acetyl-cysteine.[54] Neuronal damage is not reversed with glucose or heme therapy[201] but there is evidence that B-vitamins might support nerve regeneration.[295-297] However, to make vitamin therapy an effective treatment, studies must be done to evaluate the most efficient combination of micronutrients as well as dosage. Micronutrient therapy is not a cure for the genetic mutations that result in diminished heme biosynthesis, however, it may reduce the risks associated with these disorders and may prevent the manifestation or exacerbation of the symptoms of the porphyrias. The object of nutrition management of the porphyrias is to provide a biochemical environment that supports normal heme biosynthesis. Evidence of successful treatments highlights the need for further studies of porphyria in relation to micronutrient cofactor treatment that could shed further light on these disorders. Studies on porphyria can be difficult due to the rarity of this disease as well as the fact that not all persons with the genetic defects display symptoms. There is enough evidence to suggest that some micronutrients play a role in the porphyrias. Further research in this area is required to elucidate the importance they play. A better understanding of the heme biosynthetic pathway as well as the interconnecting pathways involving micronutrient substrates and cofactors will shed light on the pathogenesis of the porphyrias and help develop safer and more effective treatments.

CHAPTER 5. THE BIOCHEMISTRY OF THE ACUTE ATTACK

5.1 The Acute Attack

As previously discussed, most acute hepatic porphyria patients are asymptomatic with normal heme levels despite the underlying genetic mutation(s) that results in reduced enzyme activity.[147, 298] Additionally, many patients with reduced activity of a heme biosynthetic pathway enzyme produce high levels of heme precursors without ever exhibiting any toxic symptoms.[14] Because of this high rate of latent porphyria and high levels of precursors in some asymptomatic patients, it has been suggested that the genetic mutation resulting in diminished activity of heme biosynthesis pathway enzyme(s), while necessary, is not sufficient to produce the clinical symptoms of the porphyrias. For this reason it is thought that there is at least one other pathogenic factor involved in the manifestation of the symptoms of porphyria.[91, 299]

In symptomatic porphyria patients heme production is slowed resulting in reduced heme levels and excess free iron; this results in increased ALAS activity due to lack of feedback inhibition by heme. The increased activity of the initial rate-controlling enzyme in the liver leads to overproduction and increased excretion of porphyrins or porphyrin precursors, ALA and PBG, without heme production. The biochemical mechanism of the acute porphyrias thus likely includes toxic effects of over-produced heme precursors, iron accumulation, and heme deficiency. These factors explain some but not all of the acute porphyria symptoms[91] and the mechanism for the peripheral neuropathy of the acute porphyrias has not yet been described.

As previously discussed, the heme biosynthetic pathway is also dependent on a variety of micronutrients which act as cofactors. Therefore nutritional status,

deficiencies, increased demand, and imbalances of micronutrients may also play a role in the manifestation of the porphyrias. This is supported by the many symptoms of porphyria that are analogous to micronutrient deficiencies (Table 4.1).

There are many theories of what might occur biochemically during an acute attack. In this chapter, possible mechanisms will be discussed and a hypothesis for the biochemistry underlying acute porphyria will be presented.

5.2 Elevated Precursor Levels

Elevated levels of ALA have been suggested to be a factor causing acute attacks in porphyria; possibly by a direct neurotoxic mechanism, oxidative damage to neuronal and membrane structures, or through interference with neurotransmitters (see below). High levels of ALA have been suggested to result in a variety of symptoms which affect both the neurological and gastrointestinal systems[300] although the mechanism for this is still speculative.

5.2.1 Neurotoxic Precursors

Excess levels of circulating ALA and PBG are found in AIP, VP, and HCP, and it has been suggested that these heme precursors are neurotoxic resulting in the acute symptoms.[301-304] Patients with ALAD-P or tyrosemia, however, are characterised by the over-production of ALA, without an increase in PBG. As these two disorders have the same neurological symptoms as the other acute porphyrias it is unlikely that PBG plays a role in an acute attack. Although porphyrins may accumulate during an acute attack[305] it is unlikely they have neurotoxic effects as they also accumulate in the cutaneous porphyrias, which do not present with acute neurological manifestations. The ALA toxicity theory is supported by the fact that levels of ALA are increased in

all the acute porphyrias and acute attacks occur only when excretion of ALA is increased. In addition, it has been reported that the excessive levels of ALA and PBG are normalised or lowered along with a reduction of acute symptoms following intravenous heme therapy.[306] However, the ALA toxicity theory assumes that over-expressed ALA in the liver can cross the blood brain barrier (BBB).[307]

The BBB is the membrane barrier that separates the circulating blood from the brain extracellular fluid in the CNS and controls access of compounds to neurons. It limits the entry of harmful substances into the CNS while allowing transport of essential nutrients that are required for maintaining proper neuronal function to neurons. ALA and PBG appear only to cross the BBB in small amounts[308] and studies suggest the concentrations of these precursors in the brain of porphyria patients is below that required to induce neurotoxicity *in vivo*.[309, 310] A more recent review speculates that AIP patients who suffer from severe reversible posterior encephalopathy syndrome during acute attacks may have increased permeability of the BBB[304] although this is unproven. The mechanism of any transport of ALA into the brain has been suggested to be either through passive diffusion through the BBB,[311] through a peptides selective transporter,[312, 313] or through oxidative damage of the BBB through protein polymerisation and lipid oxidation.[304] There is, at this time, no conclusive evidence of ALA transport across the BBB in porphyria.

5.2.2 Oxidative Damage

Excess ALA has been reported to have toxic effects resulting in oxidative damage to lipids, protein, and DNA.[304, 314] ALA is a pro-oxidant that can undergo enolization and aerobic oxidation *in vitro* and *in vivo*, yielding oxyradicals and other pro-

oxidants.[304] Treatment of human hepatocellular carcinoma cells with ALA results in induced reactive oxygen species production, which increases with ALA concentration.[315] The major pro-oxidants that are produced after ALA administration in animal models include superoxide, hydrogen peroxide, hydroxyl radicals and 4,5-dioxovaleric acid.[304] High levels of ALA *in vitro* and in animal models result in oxidative damage to lipids, protein, and DNA as well as increasing permeability of biological membranes.[304] It is possible that ALA may increase permeability of the BBB through oxidative damage, perhaps allowing neurotoxins, including ALA, to diffuse into the CNS.

5.2.3 ALA as a GABA Mimic

Another theory for the mechanism of behind the effect ALA in the porphyrias is its structural similarity to the neurotransmitter γ-aminobutyric acid (GABA) and thus its potential to mimic and perturb GABA. GABA is the chief inhibitory neurotransmitter in the mammalian CNS. In humans it is directly responsible for the regulation of muscle tone as well as its principal role of reduction of neuronal excitability.[316] ALA has a structure similar to both GABA and glutamate (Figure 5.1). At relatively high concentrations, ALA has been suggested to inhibit GABA uptake and increase GABA efflux from rat cortical synaptosomes.[307] Studies in rat brains treated with ALA report modified release of both GABA and glutamate from central synaptosomes[317, 318] most likely through its structural similarity allowing it to act as an agonist of GABA and glutamate receptors.[318] This suggests that ALA may act as a GABA agonist which could provide a rationale for some of the CNS dysfunction in the porphyrias, but only if ALA crosses the BBB.

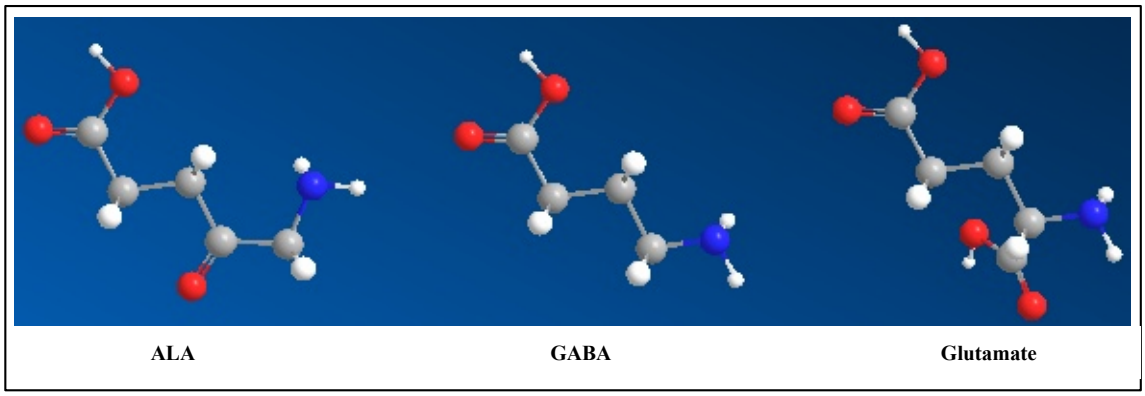

Figure 5.1 Structures of ALA, GABA and glutamate showing their structural analogies.

5.2.4 Is ALA a Factor in the Pathogenesis of Porphyria?

ALA-induced oxidative stress is likely to be a factor in the acute porphyrias, although intracellular levels of ALA and oxidative species are unknown in AIP patients. Although ALA accumulation appears necessary to trigger an acute attack, it is unlikely that ALA is the sole mechanism for triggering such attacks as injection of ALA into a healthy volunteer produced no clinical manifestations[319] and oral administration to AIP patients as well as volunteers also produced no symptoms.[320-322] There is also poor correlation between levels of blood ALA and neurological symptoms[323-325] with abnormally high levels of ALA in many symptomless AIP patients. This suggests that there must be at least one other factor involved in the symptomology of the acute porphyrias. ALA unarguably plays some role in the acute attack as levels are elevated in all of the acute porphyrias; possibly through oxidative damage of the BBB allowing the entry of neurotoxins into the CNS. However, ALA is unlikely to be the sole contributor to acute porphyric symptoms.

5.3 Heme Deficiency

5.3.1 Heme Deficiency Affects a Variety of Systems

Heme deficiency might play an important role in the pathophysiology of the acute porphyrias;[91, 326] by leading to decreased levels of hemoproteins, which results in direct or indirect effects on the nervous system. Heme is a prosthetic group in hemoglobin, myoglobin, catalases, peroxidases and the cytochromes as well as being important in regulation of its own synthesis through negative feedback inhibition of ALAS (Section 2.2.12). Deficiency of heme enzymes that are required for myriad essential processes can result in severe consequences. Effects of heme deficiency include increased enzymatic activity of heme precursor enzymes resulting in heme precursor accumulation (characteristic of the porphyrias) as well as reduced activity of essential heme enzymes.

The majority of hepatic heme (65% of total hepatic heme)[98] is utilized for the synthesis of cytochromes P450 which are required for a variety of essential reactions including the synthesis and breakdown of hormones as well as detoxification pathways. As mentioned previously, drugs and chemicals which are metabolized by the cytochrome P450 detoxification pathway trigger acute attacks. The synthesis of cytochrome P450 enzymes is up-regulated in response to toxins that are metabolized by cytochrome P450 enzymes. This up-regulation would increase the demand for heme and result in the up-regulation of ALAS leading in turn to increased heme precursor accumulation due to the 'bottleneck' of enzyme activity in the porphyric heme pathway.[327]

Another important pathway which requires a cytochrome P450 enzyme is vitamin D activation.[328] This makes vitamin D deficiency an additional risk in the context of heme depletion. As vitamin D activation also requires UV radiation from sun exposure, the porphyrias with severe photosensitive cutaneous symptoms are more susceptible to vitamin D deficiency due to avoidance of sunlight in the management of skin symptoms. There are reports of vitamin D deficiency associated with porphyria (Table 4.2).

Heme is also an essential constituent of complex IV of the electron transport chain; thus heme depletion can result in impaired ATP production, oxidative stress, and oxidative damage. ATP transports chemical energy between cells and its production is essential for the normal function of every cell. Mitochondrial complex IV activity and protein content is reduced by 95% with heme deficiency, suggesting a decrease in successful assembly and resulting in detrimental consequences.[329]

Another major heme-dependent enzyme is tryptophan pyrrolase which catalyses a step in the synthesis of NAD^+ from tryptophan.[109] Deficiency of this enzyme results in altered tryptophan metabolism with severe consequences. This pathway will be discussed in more detail as it is likely a major contributor to the biochemistry of the acute porphyric state.

5.3.2 Tryptophan Pyrrolase

Tryptophan metabolism leads to the generation of neuroactive compounds within the CNS and is highly regulated. These compounds include serotonin, kynuramines, melatonin, and tryptamine.[330] Heme is a prosthetic group of tryptophan pyrrolase, the rate limiting step of tryptophan degradation through the kynurenine pathway (Figure

5.2). Tryptophan pyrrolase has a low affinity for heme. When heme is deficient, tryptophan is shunted from the kynurenine pathway to the serotonin pathway (Figure 5.2).[326, 331] Because of this, in heme deficient states there are increased levels of tryptophan in the liver and the flux of tryptophan to serotonergic pathways.[326, 331]

High levels of tryptophan and serotonin have been recorded in the brains of heme deficient rats[326] due to altered tryptophan metabolism. Serotonin is an important neurotransmitter involved in multiple processes and is essential to nerve cells and brain function. Abnormally elevated levels of serotonin result in serotonin syndrome, a potentially life threatening disorder with symptoms that range from mild to severe (Table 5.1).[105] Serotonin syndrome is caused by high doses of serotonergenic drugs or combinations of drugs that synergistically increase serotonin levels in the CNS.[332] Some of the symptoms of this disorder (Table 5.1) are similar to those in acute porphyria and increased serotonin levels in the CNS may play a role in the pathophysiology of the acute attack.[91, 105, 307]

Table 5.1 Common Serotonin Syndrome Symptoms.

Serotonin Syndrome	
System affected	**Symptoms**
Mental	Agitation, hallucinations, confusion, hypomania, unresponsiveness or coma
Cardiovascular	Tachycardia, arrhythmia, high blood pressure
Muscular	Loss of coordination, muscle spasms (myoclonus), overactive reflexes (hyperreflexia), muscle rigidity, uncoordinated movements (ataxia), headache, seizures
Thermia	Fever, heavy sweating not due to physical activity, shivering
Gastro	Diarrhea, nausea, vomiting

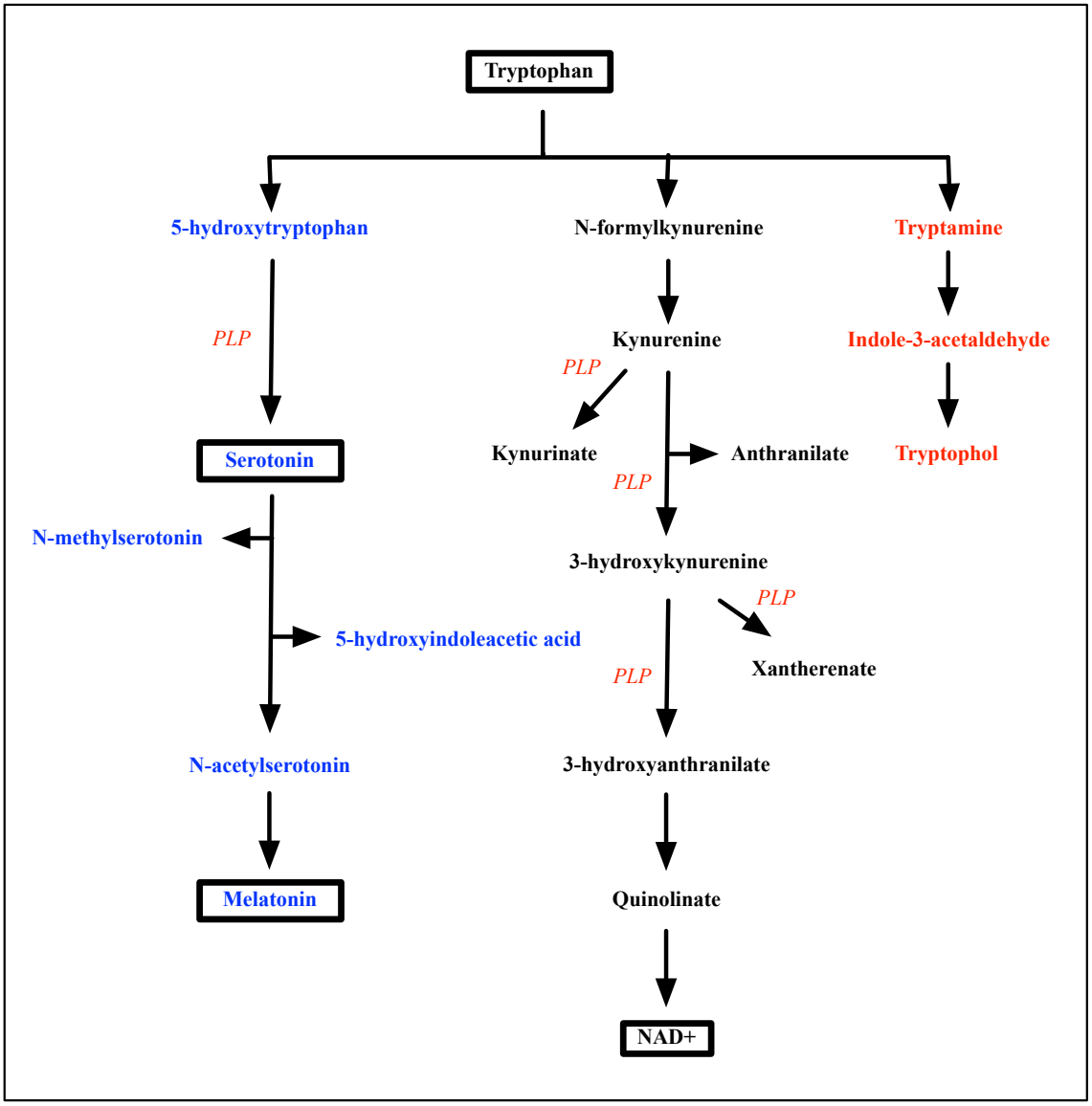

Figure 5.2 Tryptophan Metabolism Pathways.

This figure represents the major fates of tryptophan. PLP is included where it acts as essential cofactor in these pathways.

Serotonin synthesis in the brain is regulated by the transport of tryptophan across the BBB. This occurs through a competitive transport carrier shared by several large neutral amino acids including tyrosine, phenylalanine, leucine, isoleucine, and valine.[330] Serotonin itself does not cross the BBB under normal conditions and its synthesis in the brain is thus reliant on tryptophan transport.

Serotonin levels have been found to be elevated in a study of 12 AIP patients with recurrent acute attacks; yet its metabolite, melatonin (Figure 5.2), was found to be decreased.[333] After treatment of these patients with heme arginate, serotonin levels returned to normal, while melatonin levels remained reduced. Other studies show that melatonin is decreased in rats with high levels of ALA.[334, 335] GABA has been shown to have an inhibitory effect on melatonin synthesis and, as ALA has a similar structure (Figure 5.1), ALA has been suggested to also inhibit melatonin synthesis by acting as a GABA mimic.[334] As high levels of ALA occur in the acute porphyrias, it is possible that ALA interferes with the metabolism of serotonin to melatonin thus contributing to the high serotonin levels found in AIP.

As mentioned previously, ALA is a pro-oxidant, capable of producing a variety of oxyradicals. Melatonin is a powerful antioxidant and has been shown to have beneficial effects on oxidative damage, preventing cell injury[277] as well as successfully inhibiting ALA-induced oxidative damage.[131, 132] Thus, a decreased melatonin synthesis from serotonin will result not only in elevated serotonin levels but also increased oxidative damage potential.

In summary, altered tryptophan metabolism due to heme deficiency results in elevated levels of serotonin. High levels of serotonin result in a number of symptoms (Table 5.1) that are similar to some, but not all (Table 3.1) of the symptoms of acute porphyria.[91] This provides evidence that altered tryptophan metabolism plays a role in the biochemistry of the acute porphyrias, but it does not explain the peripheral neuropathy associated with acute attacks.

5.4 PLP Deficiency

As previously discussed, it has been postulated that high doses of vitamin cofactors may optimise corresponding coenzymes partially overcoming deficiencies in enzyme activity.[258] It is possible that individuals with asymptomatic porphyria remain asymptomatic through optimal nutrient status due to the major role nutrients play in heme biosynthesis as cofactors.

There are numerous reports of high doses of B-vitamins treating both acute and cutaneous porphyria symptoms, although this is not widely reported in the current literature (Table 4.3). Vitamin B6 stands out as the one supplement reported to correct the disordered biochemistry in porphyria and alleviate the symptoms of the major acute as well as non-acute porphyrias (Table 4.3). Symptoms of vitamin B6 deficiency are similar to symptoms of porphyria and include reduced immune function, neurological abnormalities, peripheral neuropathy, seizures, psychiatric disturbances, paresthesia, burning and painful dysthestias, lesions of the skin and mucous membranes, anemia and thermal sensations. The peripheral neuropathy of vitamin B6 deficiency is very similar to that found in the porphyrias[120-122] and it has been previously postulated that vitamin B6 depletion may be a factor in the neuropathy of porphyria due to its role in the first and rate limiting step of heme synthesis.[122, 336] Vitamin B6 deficiency has been reported in AIP patients,[11-13, 264] and PLP therapy has been reported to successfully alleviate the symptoms of AIP,[12] as well as CEP,[55] PCT,[32, 33] and EPP[46] symptoms. Here we will discuss the importance of vitamin B6 and its coenzyme form of PLP in heme biosynthesis and why PLP may treat all of the porphyrias.

5.4.1 Causes of Micronutrient Deficiency

"In order for vitamins to be useful to the metabolic activity of the nervous system, they must first gain entry into the tissue, where they are converted into their active cofactor form and where they interact with presumably specific apoenzyme proteins" – Dreyfus.[337]

The five possible causes of all nutrient deficiency include inadequate dietary ingestion, increased excretion, increased requirement, inadequate absorption, and/or inadequate utilization.[338]

As vitamin B6 is present in almost all foods, vitamin B6 deficiency due to insufficient dietary intake is reputed to be rare.[339] However, vitamin B6 deficiency prevalence has been reported to range from 0.6% to 68% in developed countries.[340]As there have not been many dietary studies of porphyria patients, insufficient dietary intake of vitamin B6 cannot be ruled out as a possible contributor to vitamin B6 deficiency. Insufficient dietary intake is, however, unlikely the only cause of vitamin B6 deficiency in the porphyrias.

Impaired absorption of vitamin B6 across the gastrointestinal tract and/or impaired metabolism of vitamin B6 into the active coenzyme form of PLP could also result in a vitamin B6 deficiency. Vitamin B6 exists as pyridoxine, pyridoxal, and pyridoxamine as well as their 5-phosphorylated compounds of which the major coenzyme is pyridoxal-5-phosphate (PLP) (Figure 5.3).[340] Metabolism of PLP can be impaired by a number of factors, including drugs or chemicals, impaired liver function, hereditary factors, or other micronutrient deficiencies such as riboflavin, zinc or magnesium. Zinc is required for vitamin B6 phosphorylation; riboflavin is required for conversion

to the active form of PLP; and magnesium is required for vitamin B6 uptake into tissues (Figure 5.3).

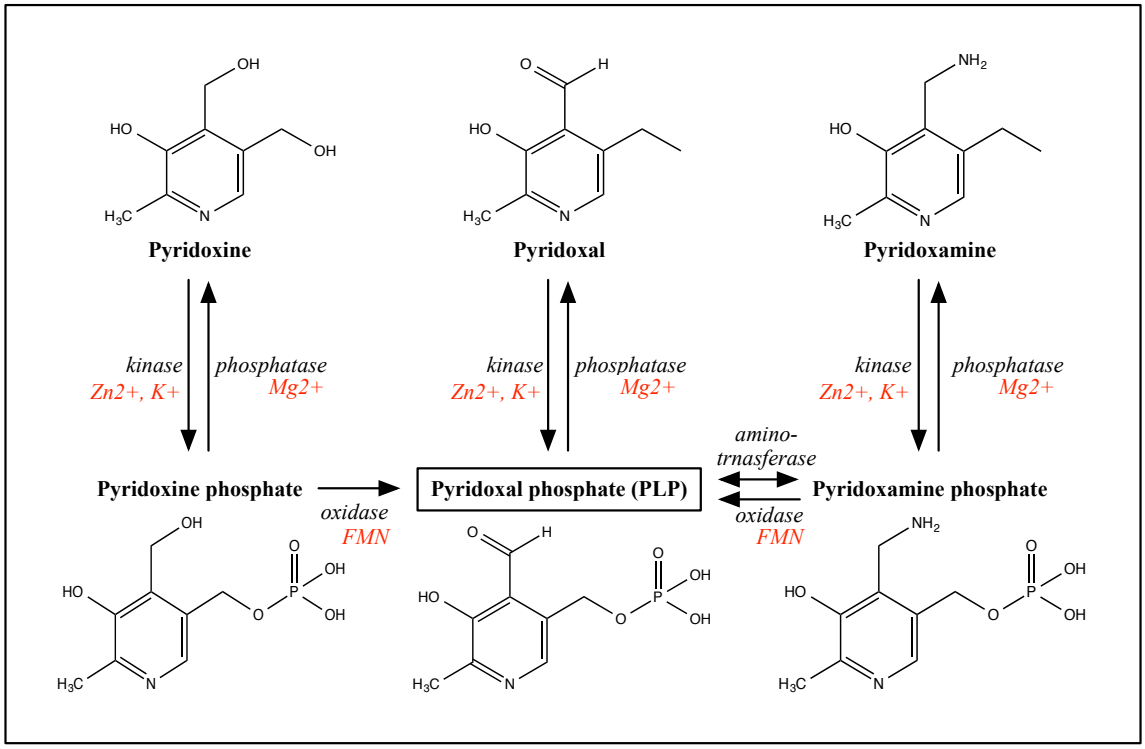

Figure 5.3 Vitamin B6 Metabolism.

The structures of the major forms of vitamin B6 and the interconversions between these forms is illustrated. Essential nutrient cofactors are shown in red. FMN, flavin mononuleotide (riboflavin).

Inadequate supply of heme to meet metabolic demands due to a partial enzymatic 'block' in the heme pathway results in the accelerated production of ALAS, the rate limiting step of heme biosynthesis. As ALAS is a PLP-dependent reaction, enhanced activity of ALAS may utilise abnormally high amounts of PLP. In a porphyric state, PLP is required and may be utilised in excess due to the demand from the up-regulated PLP-dependent ALAS enzyme. Severe vitamin B6 deficiency does not impair ALAS induction or activity in vitamin B6 (pyridoxine) deficient rats.[341] This suggests that ALAS has a high affinity for PLP in comparison to other PLP-dependent

enzymes. If this is the case, in a vitamin B6 deficient state, ALAS will be a 'high priority' enzyme and will utilise and deplete PLP from other tissues further aggravating and worsening a vitamin B6 deficiency as well as straining other PLP-dependent pathways. It takes eight molecules of ALA (and its substrates) to make each porphyrin ring. ALAS requires two bound PLP to function properly. In theory, it therefore takes 16 molecules of PLP to make each porphyrin ring. However, it is important to take into account that PLP can be recycled and thus the actual amount of PLP utilized may not completely represent the stoichiometry suggested here. However, it is easy to imagine that large amounts of PLP and other micronutrient substrates of heme are tied up in the enzymatically aberrant heme biosynthetic pathway. Therefore, the main cause of vitamin B6 deficiency in the porphyrias may be due to consumptive tissue depletion from blood and tissue due to the increased activity of ALAS.

5.4.2 The Cascading Effects of a PLP Deficiency

As discussed, vitamin B6 is a water-soluble vitamin. with pyridoxal-5-phosphate being the major coenzyme form of the vitamin and is an essential cofactor in over 300 enzymatic reactions. Due to PLP's essential role in so many enzymatic reactions, PLP deficiency has been related to a number of clinical symptoms.[9]

In a deficiency state, some PLP functions may be adequately compensated but others may not.[342] Various PLP-dependent enzymes have inherently different binding affinities with the coenzyme. Thus, those with the greatest affinity for the coenzyme are affected less than those with lower binding affinities which are the first to be affected by a PLP deficiency and the last to recover upon replenishment with the

coenzyme.[112] A few of the many detrimental effects resulting from a PLP deficiency will be discussed here.

5.4.3.1 Multiple Deficiencies

Multiple cascading deficiencies can result from a PLP deficit including, but not limited to, insulin deficiency, magnesium deficiency, zinc deficiency, and deficiency of other B vitamins. We will explore some of these cascading deficiencies resulting from a vitamin B6 deficiency here.

When a vitamin B6 deficiency is present, other micronutrient deficiencies are present as well; vitamin B6 deficiency usually occurs in combination with other B-complex vitamin deficiencies.[339] The fact that these B-vitamin deficiencies aggravate each other is an important factor when discussing the effects of any B-vitamin deficiency. Deficiency of PLP impairs vitamin B12 absorption in rats[102] resulting in the coexistence of vitamin B6 and B12 deficiency. A study on patients presenting with B12 deficiency neuropathy show riboflavin and vitamin B6 (pyridoxal) are also reduced.[127] In addition, supplementation with a single B-vitamin can induce a functional deficiency of another B-vitamin.[274] This emphasises the importance of supplementing with vitamin B-complex rather than a solitary B-vitamin.

Vitamin B6 is also crucial to Ca^{2+} and Mg^{2+} balance.[119] A PLP deficiency has also been shown to result in increased urinary Mg^{2+} excretion leading to a negative Mg^{2+} balance.[119, 343] Mg^{2+} is also an essential cofactor of over 300 enzymes and a deficiency of this mineral results in its own myriad of consequences. The presence of Mg^{2+} ions are essential in all enzymes that utilise ATP as an energy source and those that synthesise DNA and RNA from nucleotides.[344] In the context of the current

discussion it is remarkable how so many micronutrients are interconnected; changing the levels of one results in deficiencies elsewhere. Thiamine concentration has been shown to be lower in Mg^{2+} deficient rats suggesting that Mg^{2+} deficiency may inhibit thiamine-related enzyme systems,[345] while another study suggests that thiamine deficiency inhibits Mg^{2+} utilization.[346] Mg^{2+} deficiency can additionally result in a Ca^{2+} deficiency (also reported in vitamin B6 deficiency), as well as iron accumulation, and is associated with impaired vitamin B6 status as it is required to activate vitamin B6 phosphorylation (Figure 5.3). Mg^{2+} deficiency results in reduced alkaline phosphatase activity, the metalloenzyme required for uptake of PLP by tissue (Figure 5.3).[347] If a vitamin B6 deficiency leads to Mg^{2+} deficiency[119, 343] and Mg^{2+} deficiency leads to vitamin B6 deficiency,[347] a deficiency in either may result in a vicious cycle. Mg^{2+} may also protect against the mitochondrial damage promoted by ALA-generated free radicals;[348] another mechanism suggested to play a role in acute porphyria. Interestingly, hypomagnesia is common in acute porphyria attacks.[22] Could this be related to a vitamin B6 deficiency?

Another detrimental effect of PLP depletion is hyperhomocysteinemia, which is common in the porphyrias. Hyperhomocysteinemia in the porphyrias has been suggested to result from consumptive tissue depletion of PLP.[11] Deficiencies of thiamine, riboflavin, folic acid, vitamin B12, and biotin, in addition to vitamin B6 are associated with central nervous system abnormalities.[126] Santhosh-Kumar et al.[349] has suggested that the neuropsychiatric manifestations of some of these B-vitamin deficiencies may be due to disrupted homocysteine catabolism in the brain. Vitamin B6, folate and vitamin B12 supplemented together have resulted in better results than

any of the afore mentioned B-vitamins on their own in lowering homocysteine levels.[274]

The interactions between vitamin B6, other B-vitamins, as well as other micronutrients are overwhelmingly complex and it is easy to imagine how a deficiency of one will result in deficiencies of many others.

5.4.3.2 PLP as an Essential Coenzyme

"PLP is one of nature's most versatile cofactors" - Schneider et al.[350]

PLP is an essential coenzyme to a wide variety of enzymatic reactions including transamination, decarboxylation, racemization, amine oxidation, aldol reactions, cleavage, deamination, dehydration, and synthesis. It is also involved in fatty acid metabolism and nucleic acid synthesis as well as membrane function and stability. It is notable that many pathways are affected by a PLP deficiency; only a few of them already known to play a role in porphyria will be briefly mentioned.

We already know that PLP is an essential cofactor for the rate-limiting ALAS enzyme of heme synthesis and that mutations of this enzyme that alter PLP-binding to the enzyme cause XLSA.[116] PLP has also been suggested to play a role in heme synthesis as a cofactor of ferrochelatase, the final enzyme of the heme pathway. PLP deficiency causes protoporphyrinemia with correlation between ferrochelatase activity and PLP concentration.[96] In addition, PLP deficiency is shown to stimulate increased iron deposits[117] commonly found in porphyrias which leads to increased oxidative damage.

Vitamin B6 is also important in the maintenance of $Na^+$and K^+ balance[118] and water homeostasis has been reported to be impaired in a PLP deficient state, through a study

involving vitamin B6 deficient rats.[118] Na^+ levels were found to be elevated and K^+ levels reduced in the serum of the vitamin B6 deficient rats suggesting a role of B6 in the maintenance of Na^+ and K^+ balance. Another study suggests a delayed diuretic response to water loading in rats maintained on diets deficient in either pyridoxine or pantothenate.[351] Inappropriate secretion of antidiuretic hormone is a common complication of the acute porphyrias manifesting as dilution hyponatremia and hypokalemia and increased total body fluid.[228, 230-233, 245, 352, 353] This may be due to vitamin B6 deficiency.

5.4.3.3 Tryptophan - a Common Issue

Other important PLP reactions are the conversion of tryptophan to both niacin and serotonin (Figure 5.2). PLP is an essential cofactor of kynurinase, an enzyme in the tryptophan-niacin pathway. Kynurinase has a low binding affinity with PLP and its activity is highly impaired by low levels of PLP. This is the basis of the tryptophan load test that has been used to measure PLP status. PLP deficiency therefore impairs kynurinase activity and disrupts tryptophan metabolism resulting in a number of detrimental biochemical sequelae. Kynurinase impairment leads to diminished niacin synthesis and niacin deficiency. Pellagra is the classical niacin deficiency syndrome and can be fatal if untreated. Vitamin B6 deficiency has been shown to induce niacin deficiency[120] and there are reports of pyridoxine pellagra.[354] Some of the symptoms of pellagra resemble those of the cutaneous porphyrias. A niacin deficiency could possibly aggravate the known role that porphyrins play in the skin manifestations in these porphyrias. Kynurenase activity has been reported to be reduced in patients with PCT[355] possibly due to diminished vitamin B6 status.

A block in tryptophan-niacin metabolism due to kynurinase impairment caused by a vitamin B6 deficiency also leads to increased xanthurenic acid levels.[356] A computational model of tryptophan modeled a vitamin B6 deficient state and predicted increased serotonin as well as xanthurenic acid levels correlated with diminished PLP levels.[357] Xanthurenic acid readily binds with insulin impairing its function by making it less effective.[358] Insulin deficiency has been reported in PLP deficiency and is believed to be due to reduced pancreatic synthesis of the hormone.[113] Vitamin B6 deficiency resulting in reduced insulin availability[359] may be an additional factor in the onset of diabetes often seen in porphyria (Section 4.2). This may contribute to the biochemical basis of the disordered carbohydrate metabolism found in the acute porphyrias and play a part in the "glucose effect" as insulin inhibits ALAS1 induction.

5.4.3.4 GABA

A previously discussed theory behind the acute attack in porphyria is the structural similarity of ALA to GABA and thus its ability to mimic GABA. GABA is largely formed from glutamate in the presence of glutamate decarboxylase which is a PLP-dependent enzyme. Vitamin B6 deficient states result in lower levels of GABA production and as GABA mediates post-synaptic inhibition, failure of its synthesis can lead to seizures.[360] Therefore, in a vitamin B6 deficient state, ALA may have reduced competition from GABA potentiating the ability of ALA to act as a GABA agonist (Section 5.2.3).

5.4.4 The PLP Deficit

Nutrients play an important role in heme synthesis (Table 2.1). Deficiencies of essential vitamins and minerals can provide a plausible explanation for why some

patients with a porphyric mutation may experience an acute attack in response to a trigger, while other patients with the same mutation may remain asymptomatic. Between 70-90% of the carriers of a genetic mutation associated with a porphyria remain latent or asymptomatic;[147, 298] thus an additional factor is required in the manifestations of this disorder. Additionally, many patients with deficiency in activity of a heme biosynthetic enzyme produce high levels of heme precursors without ever exhibiting symptoms.[14] Because of this high rate of latency and high levels of precursors in some asymptomatic patients, it has been suggested that the genetic mutation resulting in a diminished heme biosynthesis pathway enzyme, while necessary, is not sufficient to produce the clinical symptoms and that there is at least one other pathogenic factor involved in the manifestation of the symptoms of acute porphyria.[91]

Rapid tissue consumption of PLP leading to depletion of its *in vivo* concentration due to the increased activity of the initial steps of the heme biosynthetic pathway in the acute porphyrias is hypothesized to be one of the precipitating factors in the pathogenesis of the acute attack.[122, 336] Terroine and Adrian[361] demonstrated that deficiency of PLP also results in reduced concentration of riboflavin, pantothenic acid and nicotinamide in the liver. Terman and Farmer,[362] suggest that it is deficiencies of these B-vitamins in conjunction with vitamin B6 that result in the clinical and pathological manifestations of AIP. As seen above and in the heme biosynthesis chapter (Chapter 2), these cofactors have interconnected pathways and their levels correlate together. Emphasis must thus be placed on the fact that a PLP deficiency is correlated with changed levels of other key micronutrient cofactors. If PLP deficiency

plays a role in porphyria it will be due to a combination of other micronutrient deficiencies as well.

5.5 Discussion and Hypothesis

There have been several hypotheses to explain what occurs during an acute attack of porphyria, the most popular being high levels of ALA and heme deficiency. Another hypothesis that has been suggested but is often disregarded[307] in the current literature is a consumptive tissue depletion of vitamin B6 by increased activity of ALAS in a porphyric state. This theory is often disregarded due to lack of evidence of successful pyridoxine supplementation.[363] However, the most cited paper found through a literature review that suggests no effect of pyridoxine supplementation in the porphyrias used only a single dose of 100 mg of pyridoxine orally on two patients.[363] The authors reported increased plasma pyridoxine values that rapidly decreased to subnormal values again with no clinical improvement in these two cases. This same paper reports PLP deficiency in a study of 21 AIP patients and reported a generally lower PLP concentration during acute attacks, which supports the PLP-deficit theory. In another report published at a similar time, treating AIP patients with vitamin B6 (pyridoxine) 400 mg/day resulted in some improvement after 3 months,[12] possibly due to the higher dose and continuous dosage. One must also consider, that the pyridoxine form of vitamin B6 may not be being converted effectively into PLP due to co-existent B-vitamin deficiencies. Therefore the coenzymated form of the vitamin may present with better results by overcoming any potential metabolic obstacles to the conversion of the vitamin to its cofactor form.

In conclusion, the acute porphyric attack is most likely due to a combination of factors including toxic effects of ALA, deficiency of the heme enzyme tryptophan pyrrolase leading to increased serotonin levels, as well as consumptive tissue depletion of essential micronutrient cofactors. These factors are intimately connected. Heme deficiency results in many altered processes which require heme components including that of tryptophan pyrrolase, resulting in a deficiency of this enzyme. Tryptophan pyrrolase deficiency leads to elevated levels of circulating serotonin, which is in turn not converted to melatonin due to inhibition, possibly through the high levels of ALA which acts as a GABA agonist, leading to serotonin toxicity symptoms. This secondary 'serotonin syndrome' could also be initiated or enhanced by a vitamin B6 deficiency due to the important roles of vitamin B6 (PLP) in tryptophan metabolism. ALA may, as previously suggested, have some oxidative effect in porphyrias although this cannot be the sole mechanism of porphyria as levels do not correlate with acute attacks,[323-325] and oral administration of ALA will not cause an acute attack.[319, 320, 322] However, oxidative damage by ALA may disrupt the BBB resulting in increased permeability, perhaps leading to elevated serotonin levels in the CNS. Elevated serotonin levels in the CNS account for most of the symptoms of acute porphyria.[91] However, the only mechanism that accounts for the peripheral neuropathies is essential micronutrient deficiencies; in particular a vitamin B6 deficit. Could this be the final piece in the puzzle of the biochemistry of the acute attack? A vitamin B6 deficiency on its own causes myriad cascading effects and leads to altered metabolism of essential processes as well as aggravation of other vitamin and mineral deficiencies resulting in additional symptoms. In summary, heme deficiency resulting in altered tryptophan metabolism in addition to ALA over-accumulation can explain

all of the porphyric symptoms other than the peripheral neuropathies.[91] Peripheral neuropathy is proposed to be due to a vitamin B6 deficit.

To gain a better understanding of what occurs biochemically during an acute attack of porphyria, further investigation into the heme biosynthetic pathway as well as the many interconnected pathways that may also contribute is essential.

The role of PLP in tryptophan metabolism has recently been investigated through the creation of a mathematical model of tryptophan metabolism based on enzyme kinetics of published literature.[357] This model was able to calculate the flux of metabolites and simulate a vitamin B6 deficiency resulting in increased serotonin and decreased tryptophan, which is known to occur *in vivo*. This was done through calculating response of enzymes to varying PLP concentrations rather than changing enzyme activities directly. This successful modeling of tryptophan metabolism suggests that a similar model of the heme biosynthetic pathway may also be able to measure changes with varying PLP concentrations and thus test the hypothesis.

Hypothesis

It is hypothesized that a PLP deficit is induced or exacerbated in the porphyrias by the up-regulated activity of the early steps of the heme biosynthetic pathway and that this PLP deficit and the subsequent cascade of biochemical insults resulting from this deficit is the precipitaiting factor required in addition to the genetic mutation for the manifestation of the symptomology of the acute porphyrias.

CHAPTER 6. BUILDING THE MODEL

6.1 Introduction to Biomodeling

"Modeling allows one to do "what-if" experiments leading to new hypotheses that can later be put to test experimentally" – Mitchell and Mendes.[367]

In the previous chapter we have seen that the flux of metabolites through the heme biosynthetic pathway are relevant to porphyria. The investigations into the biochemistry of the acute porphyrias, and the resulting proposed hypothesis presented in Chapter 5, demonstrates the requirement for further investigation of this heme biosynthetic flux. In order to investigate the hypothesis, theoretical tools are needed to improve understanding of these complex pathways. Systems biology and computational modeling provide potential tools for the modeling and investigating of the heme biosynthetic pathway in the context of porphyria.

Systems biology, the study of complex interactions within biological systems, is becoming an increasingly popular and useful area of research and is expanding our knowledge of biochemical processes.[364-366] In particular, the investigation of complex biological systems through computational and mathematical modeling provides methodology and potential for advances in biomedical and biologically scientific research.[365, 367]

Biochemical networks are sets of reactions linked together by common substrates and products.[368] Computational models of biochemical pathways and networks require rate laws and enzyme kinetic data to model each reaction. Due to this requirement and increasing demand for this data, enzyme kinetics, a century old area of research, is regaining popularity as a key area of biochemical research.[368] Quantitative modeling

of biochemical networks using enzyme kinetic data allows theoretical investigations into metabolic pathways. Pathway flux as well as metabolic interconnections can be investigated and later tested experimentally.

A computational model of the heme biosynthetic pathway would considerably advance the understanding of the complex interrelationships and regulation of heme synthesis. As the altered biochemistry of the acute porphyrias is not fully understood, a model of the heme biosynthetic pathway could be used to not only investigate the biochemistry of the porphyrias and test current hypotheses but also to develop new hypotheses for the pathophysiology of acute attacks. As the heme biosynthetic pathway is inherently dependent on other pathways, such as cofactor synthesis and the TCA cycle (Table 2.1), such a model would ideally be complex enough to include these interconnected pathways.

In order to build a realistic model which can be used to mimic the heme biosynthetic pathway, rate laws as well as kinetic data for the parameters of each of the enzymes and individual steps of heme biosynthesis are required. For the model to be relevant for investigations into porphyria biochemistry in humans, the values used should preferably be derived from human studies. The parameters entered into the model will provide the best results if they have been measured under similar conditions and, if possible, from the same tissue source. Mathematical and computational models of systems are not complete representations of their corresponding living system, and detailed knowledge of the kinetic parameters involved are essential for a model to mirror its living system. The closer these parameters are to that in real systems the more meaningful, in an *in vivo* context, the result. These parameters are usually derived from *in vitro* experiments and thus may not compare completely with *in vivo*

situations. Nonetheless models can still give us valuable information about fluxes and interactions within systems.

The model presented in Chapter 7 will utilise data from each of the individual steps of the heme biosynthetic pathway to measure metabolite flux through the pathway. This chapter will discuss enzyme parameters of the heme biosynthetic pathway enzymes compiled from an exhaustive literature review and the parameter values that have been used as the basis for parameterising the metabolic model of the heme biosynthetic pathway which is presented in Chapter 7. The data presented here will compliment the background of heme biosynthesis described in Chapter 2.

6.2 Building the Model

6.2.1 Kinetic Background

The rate of a reaction is determined by substrate concentration, enzyme concentration, concentrations of inhibitors and activators and by specific kinetic parameters that measure the rate of the reaction.[369] A simple one step irreversible enzyme catalysed reaction can be described by the Michaelis-Menten (M-M) model that assumes a simple 2-step reaction: (1) substrate binds to enzyme, (2) substrate is converted to product and released. The irreversible form of this simple reaction is shown in Equation 6.1.

Equation 6.1: $A \rightarrow B$

$$v = \frac{V_{max} \cdot A}{K_M + A}$$

Where V_{max} represents the maximum rate achieved by the reaction at saturating substrate concentrations, and K_M characterises the interaction between enzyme and substrate (A) and corresponds to the substrate concentration at which the reaction is at 50% of V_{max}. K_M is an inverse measure of affinity between the substrate and enzyme; a lower K_M represents a higher affinity between the substrate and enzyme. M-M kinetics describe enzyme reactions exhibiting simple hyperbolic kinetics rather than cooperative kinetics found in regulated enzyme activity.

6.2.2 Literature Search

All eight of the enzymes involved in heme synthesis have recorded literature values for V_{max} and K_M. It is assumed that all the enzymes obey simple M-M kinetics unless otherwise stated (Equation 6.1). For simplicity the literature search was limited to these two parameters in order to have consistency between the different enzymes; the turnover number (kcat), specific activity, and other kinetic parameters were ignored.

Finding the relevant data for each of the enzymes involved in the heme biosynthetic pathway proved to be an arduous task. It was a challenge finding the data required to create a heme biosynthesis model that is relevant for humans and particularly relevant for the porphyrias. Although there are many databases available today providing kinetic parameters and enzyme data, there are still many gaps in the data available for heme enzymes and extensive investigation into the current body of literature was required. For each enzyme, BRENDA (BRaunschweig ENzyme DAtabase),[370] which is the kinetic scientist's "bible" for enzyme information, was the starting point for

gathering kinetic parameters. As the BRENDA database did not include all of the parameters required for modelling each of the heme enzymes, an expanded search in PubMed[371] and other databases was also conducted.

This review will present some kinetic parameters of heme enzymes found in the literature; this augments the background of heme biosynthesis presented in Chapter 2, and creates a groundwork for the construction of a computational model of the heme biosynthetic pathway.

6.2.2.1 Aminolevulinate Synthase (ALAS)

$$glycine + succinyl\text{-}CoA \rightarrow ALA + CoA + CO_2$$

ALAS (EC 2.3.1.37), as discussed in Section 2.2.5, is a homodimer and catalyses the first step of the heme biosynthetic pathway. This enzyme is believed to be rate-limiting for the pathway as it has the lowest activity of all of the enzymes of the heme biosynthetic pathway.[372] The enzyme catalyses the condensation reaction between succinyl-CoA and glycine to produce ALA with the release of CoA and CO_2.

ALAS requires two PLP cofactors; it has two active sites per dimer which each bind a PLP molecule symmetrically.[116, 373] The reaction mechanism occurs via ordered binding of glycine which forms a PLP-glycine complex followed by binding of succinyl-CoA which donates a succinyl group via PLP.[72, 374]

As discussed throughout earlier chapters, ALAS1 activity is tightly regulated through feedback inhibition by heme. The mechanism of feedback inhibition may be at the transcriptional, translational and transport levels and is not fully understood.[73, 375] As

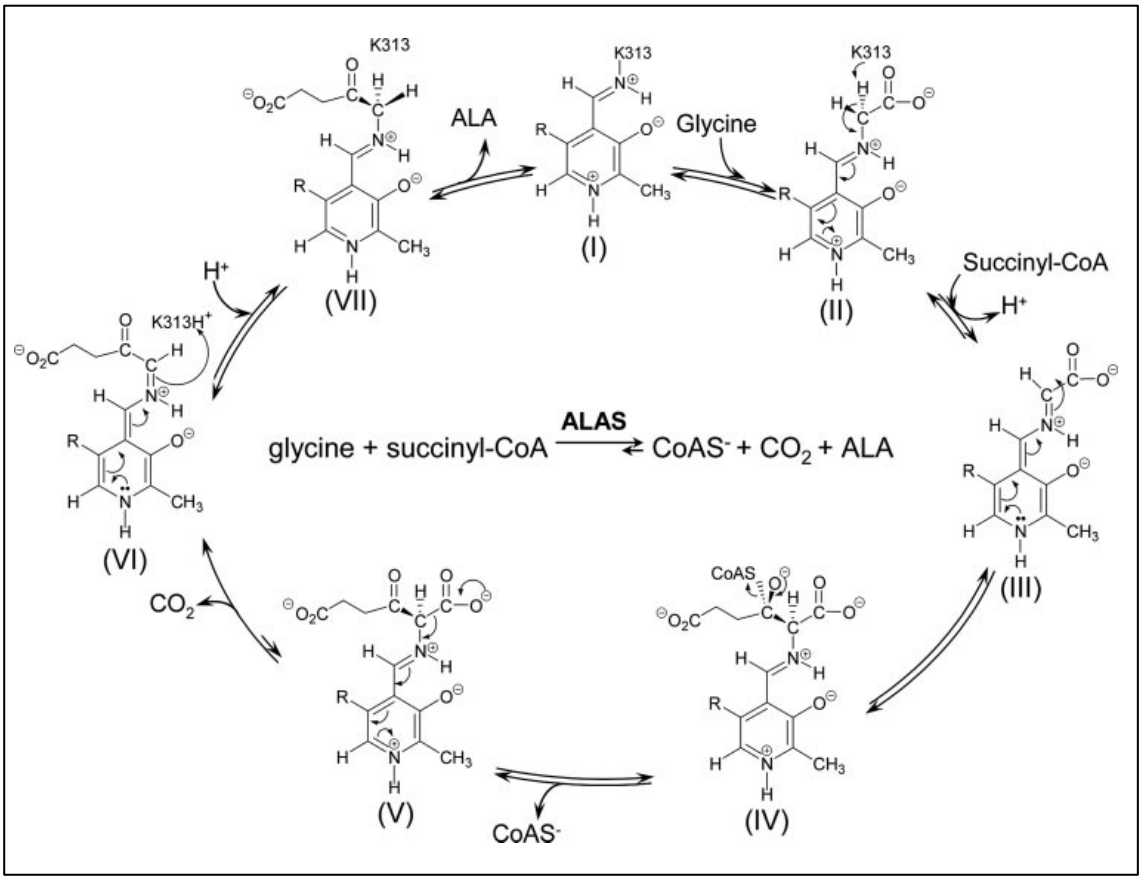

Figure 6.1 ALAS Mechanism.

Suggested mechanism for the PLP-dependent ALAS reaction reproduced from Hunter and Ferreira.[72]

regulation of ALAS is not completely understood, feedback inhibition of the enzyme by heme was not considered at this stage and M-M kinetics was assumed as a simplifying assumption for the development of the initial model.

A stepwise, ordered bi uni mechanism has been suggested for ALAS:(Hunter and Ferreira 2007;2011)

$$E + G \leftrightarrow EG + SCoA \leftrightarrow EGSCoA \leftrightarrow EQ \leftrightarrow EALA1 \leftrightarrow EALA2 \leftrightarrow E + ALA$$

However, even this mechanism is not complete as it does not include PLP binding. For the first simple model of heme biosynthesis, 'Stage 1' (which will be presented in

Chapter 7), a single, irreversible bi reaction was assumed for simplicity, rather than the more complicated six step reaction mechanism above (Equation 6.2).

Equation 6.2: $A + B \rightarrow C$

$$v = \frac{V_{max} \cdot A \cdot B}{K_{Ma} \cdot K_{Mb} + A \cdot K_{Mb} + B \cdot K_{Ma} + A \cdot B}$$

The K_M and V_{max} parameters found from the literature for this enzyme through the literature search are recorded in Table 6.1. All parameters listed in Table 6.1 are derived from studies of erythrocytes and values may be different than those from hepatocytes. It must be considered that parameters from different sources may vary, and kinetic values from erythrocytes may be more relevant to investigations into the erythropoietic porphyrias rather than the hepatic porphyrias.

Gong et al.[377] also recorded dissociation constants for glycine, pyridoxal-5-phosphate, and aminolevulinate at 22 mM, 1.6 μM and 25 μM respectively. These parameters may be useful for creating a subsequent model which includes reversible reactions. However, it should be noted these values are measured in murine rather than human tissue.

The last three rows in Table 6.1 show values from human models. Ducamp et al.[77] recorded a K_M value for succinyl-CoA that was significantly lower then the Bishop et al. values.[78, 378] This could possibly be due to different assay conditions as they used a lower temperature and pH. For the 'Stage 1' model of heme synthesis in the following chapter, the average of the K_M and V_{max} of references 28 and 360 were used (Table 7.2).

Table 6.1 K_M and V_{max} Parameters Found for ALAS.

Organism	**Location**	V_{max} **Gly (µmol/l*min)**	V_{max} **S-CoA (µmol/l*min)**	K_M **Gly (µM)**	K_M **S-CoA (µM)**	K_M **PLP (µM)**	**pH**	**°C**	**Reference**
Mouse	Erythrocyte	—	—	51000	55	—	7.2	37	376
Mouse	Erythrocyte	—	—	23000	2.3	—	7.2	30	377
Human	Erythrocyte	—	—	15000	4.3	—	7.2	30	77
Human	Erythrocyte	5.2	5.15	9300	40.7	0.0215	7.4	37	78
Human	Erythrocyte	4.975	—	10200	47	0.0278	7.4	37	378

Recorded values for K_M and V_{max} parameters found for ALAS in the literature.

6.2.2.2 Aminolevulinate Dehydratase (ALAD)

$$ALA + ALA \rightarrow PBG + 2\ H_2O$$

The next step in heme biosynthesis involves ALA transportation into the cytosol (parameters unknown) where it is converted into PBG. ALAD (EC 4.2.1.24) then catalyses the condensation and cyclisation of two molecules of ALA to form PBG.[379]

This enzyme, found in the cytosol, exists primarily as a homo-octamer and requires zinc ions for activity with the ability to bind up to eight zinc ions.[380, 381] However, it is suggested that only four zinc ions are required for optimal activity (reference). Zinc ions may play a role as a facilitator for the binding and reactivity of the active site towards ALA or through maintaining the stability of the enzyme structure.[382]

ALAD also exists as a low activity hexamer at higher pH levels,[383] and it is thought that some mutations that are the underlying cause of ALAD-P may result in stabilisation of this hexameric form.[384]

The enzyme obeys M-M kinetics and the irreversible M-M equation (Equation 6.1) was used for simplicity in the 'Stage 1' model of heme synthesis (Chapter 7). In later models, a more complicated rate law will better represent the stoichiometry of this reaction.

Values for K_M and V_{max} for ALAD from the literature search are shown in Table 6.2. For the 'Stage 1' model, K_M and V_{max} values from Anderson and Desnick[385] were chosen as they reported a usable V_{max} from human tissue (Table 7.2).

Table 6.2 K_M and V_{max} Parameters Found for ALAD.

Organism	Location	V_{max} (µmol/l*min)	K_M (µM)	pH	°C	Reference
Bacillus subtilis	—	—	118	9.0	37	382
Bovine	Liver	—	273	6.8	37	382
Mouse	Hepatic	5.95	320	6.4	37	386
Human	Erythrocyte	1.26	270	6.8	37	385
Human	Whole blood	—	287	6.8	37	380
Human	Plasmid	—	200	6.2-9.2	37	383

Recorded values for K_M and V_{max} parameters found for ALAD in the literature.

6.2.2.3 Porphobilinogen Deaminase (PBGD)

$$PBG + PBG + PBG + PBG \rightarrow HMB + 4\ NH_3$$

PBGD (EC 2.5.1.61), the third enzyme in the heme biosynthetic pathway which converts four PBG molecules into HMB, utilises an unusual dipyrromethane cofactor. This enzyme is a monomer found in the cytosol.

There is evidence that suggests that folate may also be involved in this reaction as a non-essential activator of PBGD.[83] This study in rat liver suggests that folate produces an increase in V_{max} without altering the K_M value.[83] This could be significant when investigating the mechanism of folate treatment of the acute porphyrias as it has been suggested that high doses of coenzymes may correct and treat enzyme deficiencies that correspond to metabolic disorders.[258]

There is also feedback inhibition of this enzyme by coproporphyrinogen and protoporphyrinogen in HCP and VP.[93] These porphyrinogens, when added to PBGD in control lymphoblasts, decrease PBGD's V_{max} and result in sigmoidal PBGD substrate-velocity curves.[93] Incorporating details of this feedback inhibition will be

important in the development of computational models of heme synthesis for investigation of HCP and VP.

PBGD utilises a covalently bound dipyrromethane cofactor, which is the result of autocatalytic coupling of PBG as a primer. Another four molecules of PBG are then linked in a head-to-tail fashion to the dipyrromethane to form a protein bound linear chain of six PBG units forming a hexapyrrole. Hydroxymethylbilane (four PBG) is then cleaved off leaving the dipyrromethane cofactor still attached to the enzyme.[56] This enzyme's kinetics are complex due to its quirky chemistry. A study of the enzyme in *E. coli* suggests that PBG first binds the dipyrromethane (PBG-PBG) cofactor and then forms a complex with each of the next PBG molecules through ES, ES2, ES3, and ES4 states and then releases HMB leaving the cofactor still bound.[387] This can be summarized as follows:

$$E + D \rightarrow ED \rightarrow EDS \rightarrow EDS2 \rightarrow EDS3 \rightarrow EDS4 \rightarrow ED + P$$

Where D is the cofactor dipyrromethane, S is the substrate (PBG), and P is the product (HMB). From a study in rat kidney,[388] Noriega proposed the best rate law to fit the experimental data was Equation 6.3:

Equation 6.3:

$$v = \frac{V1[S] + V2[S]2}{1 + K1[S] + K2[S]2}$$

They reported the apparent kinetic constants: V1 = 1017.41 pmol/min mg, K1 = 2.08 ± 0.01 μM, V2 = 86.10 ± 0.19 pmol/min mg, K2 = 0.102 ± 0.003 μM[388]. A separate V_{max} and K_M was also recorded as 439.73 ρmol/min/mg and 0.855 μM respectively. However, the complexity of this rate law was not appropriate in the 'Stage 1' model and again M-M irreversible kinetics was assumed as there are readily available K_M

and V_{max} values recorded in the literature (Table 6.3). More sophisticated models of the heme biosynthetic pathway will require the incorporation of a more accurate rate law.

V_{max} and K_M values for this enzyme from the literature are recorded in Table 6.3. Values measured from AIP patients are highlighted in blue. Data from Bustad et al.[389] was chosen for the 'Stage 1' model in Chapter 7 as this author also reported values in AIP patients which can be compared to the "normal" values during model investigations.

Table 6.3 K_M and V_{max} Parameters Found for HMBS.

Organism	**Location**	V_{max} **(µmol/l*min)**	K_M **(µM)**	**pH**	**°C**	**Reference**
E. coli	—	—	7.0	8.3	20-25	390
Rat	Erythrocyte	—	15.3	8.0	37	391
Rat	Kidney	—	0.855 (app)	8.2	37	388
Human	Erythrocyte	—	8.9	8.5	37	93
Human	Erythrocyte	—	6	6.8	37	392
Human	—	0.105 (0.051 AIP)	48 (1579 AIP)	8.2	37	389
Human	Erythrocyte	—	5.9 (app)	8.1	37	393
Human	Erythrocytes	0.693 (0.325 AIP)	12.3 (6.2 AIP)	7.4	37	394
Human	Erythrocytes	0.000035	77	7.4	37	395

K_M and V_{max} parameters found for hydroxymethylbilane synthase (HMBS). Values highlighted red were measured in AIP patients; (app) indicates an apparent parameter.

6.2.2.4 Uroporphyrinogen Synthase (UROS)

$$HMB \rightarrow uroporphyrinogen\ III + H_2O$$

UROS (EC 4.2.1.75) is a monomer also found in the cytoplasm. It catalyses the rapid closure of the linear HMB to form the first porphyrin ring of the pathway uroporphyrinogen III (Figure 2.7).

A study in rat hepatocytes suggests that folate may also affect the UROS reaction.[87] Further studies into this possible mechanism may shed new light on the effects of this vitamin on porphyria.

UROS has a higher rate of activity then PBGD, HMB never accumulates *in vivo* and PBGD and UROS work together to form uroporphyrinogen III exclusively.[396] For this one-substrate, one-product reaction, M-M irreversible kinetics (Equation 6.1) was assumed. K_M and V_{max} values from the literature are shown in Table 6.4. For the 'Stage 1' model, values from Cunha et al.[397] and Omata[398] were used as the lower K_M values utilised HMB faster which, as previously stated, never accumulates *in vivo.*

Table 6.4 K_M and V_{max} Parameters Found for UROS.

Organism	Location	V_{max} (µmol/l*min)	K_M (µM)	pH	°C	Reference
Human	Liver	0.000225	1.01	7.4	37	398
Human	Erythrocyte	—	5.0-20.0	7.4	37	399
Human	Erythrocyte	—	1.5	8.2	37	400
Human	Erythrocyte	—	0.15	7.4	37	397
Human	Erythrocytes	0.000023	51	7.4	37	395

K_M and V_{max} parameters found for UROS from the literature.

HMB is extremely unstable, and undergoes rapid non-enzyme catalysed cyclisation to form uroporphyrinogen I. However, under physiological conditions there is normally

an excess of UROS capacity and little of the I isomer forms.[3] The non-enzymatic conversion of HMB was not included in the 'Stage 1' model.

6.2.2.5 Uroporphyrinogen Decarboxylase (UROD)

$$uroporphyrinogen\ III \rightarrow coproporphyrinogen\ III + 4CO_2$$

UROD (EC 4.1.1.37), the fifth enzyme of the pathway, is a dimer and also resides in the cytoplasm. It forms coproporphyrinogen III from uroporphyrinogen III as well as coproporphyrinogen I from uroporphyrinogen I.

UROD catalyses the stepwise decarboxylation of the four acetate substituents on the beta carbon of uroporphyrinogen III to form coproporphyrinogen III via hepta-, hexa-, and penta-carboxylate intermediate forms.[401] This enzyme has complex kinetics as all of these intermediates have reported K_M's for the enzyme[402] and can each act as a substrate as well as accumulate as their corresponding porphyrins in porphyria (Figure 2.9).

This enzyme is also capable of metabolising uroporphyrinogen I to coproporphyrinogen I via hepta-, hexa- and penta-carboxylate intermediates as well, further complicating the enzyme's already complex enzymes kinetics. Coproporphyrinogen I, however, plays no further role in the heme pathway,[401] and in the 'Stage 1' model (Chapter 7) this branch of the heme biosynthetic pathway is not considered.

K_M and V_{max} values from the literature for both the I and III porphyrinogen isomers are shown in Table 6.5. Warby et al.[403] reports a much higher K_M and lower V_{max} than

Table 6.5 K_M and V_{max} Parameters Found for UROD.

Organism	Location	V_{max} I (µmol/l*min)	V_{max} III (µmol/l*min)	K_M I (µM)	K_M III (µM)	pH	°C	Reference
Rats/mice	Liver	0.096	0.19572	2.1	2.7	6.8	37	404
Human	Leukocytes	0.0000732	0.000186	38.1	10.8	6.8	37	403
Human	Erythrocytes	0.03325	0.06425	1	0.4	6.75	37	402
Human	Erythrocytes	—	—	0.8	0.35	6.8	37	405

K_M and V_{max} parameters found for uroporphyrinogen synthase (UROS). Values for both the I and III isomers are included.

the other investigations reported; this could possibly be due to the differences in tissue source (leukocytes) and or the higher pH conditions than the other references from human sources. Data from de Verneuil et al.[402] was used in the 'Stage 1' model.

6.2.2.6 Coproporphyrinogen Oxidase (CPOX)

$$coproporphyrinogen\ III + O_2 + 2H^+ \rightarrow protoporphyrinogen\ IX + 2CO_2 + 2\ H_2O$$

Coproporphyrinogen III is then transported back into the mitochondria (transport parameters unknown) where it can be ultimately formed into heme. CPOX (EC 1.3.3.3), the sixth enzyme of the heme biosynthetic pathway, is found back in the mitochondria and is a homodimer. Protoporphyrinogen IX is formed from coproporphyrinogen III via oxidative decarboxylation.

CPOX is specific to coproporphyrinogen III. Coproporphyrinogen I cannot be utilised as a substrate for this enzyme and, as previously stated, the latter plays no further role in the pathway.

CPOX may require copper for optimal activity[91] although some studies suggest otherwise.[92] Copper will not be considered in the 'Stage 1' model (next chapter). The reported kinetic parameters from the literature are displayed in Table 6.6; the parameter values are relatively consistent from the different sources. Data from Li and Woods[406] although derived from liver studies, was used for the 'Stage 1' model because this source provides a recorded V_{max} value that was compatible with the model. Parameters derived from erythrocytes was not an option for modeling this enzyme as none of the data found through the literature search was based on erythrocyte studies (Table 6.6).

Table 6.6 K_M and V_{max} Parameters Found for CPOX.

Organism	Location	V_{max} (µmol/l*min)	K_M (µM)	pH	°C	Reference
Human	Plasmid	—	0.6	8.0	37	92
Human	Liver	0.004176	0.291	7.2	37	406
Human	Plasmid	—	0.68	7.2	37	407
Human	Lymphocytes	—	0.21	7.2	37	408

K_M and V_{max} parameters found for coproporphyrinogen oxidase (CPOX).

6.2.2.7 Protoporphyrinogen Oxidase (PPOX)

$$protoporphyrinogen\ IX + 3O_2 \rightarrow protoporphyrin\ IX + 3H_2O_2$$

PPOX (EC 1.3.3.4) is a dimer and a mitochondrial enzyme found in the inner membrane. It catalyses the conversion of protoporphyrinogen IX into protoporphyrin IX in a three-step, six-electron oxidative reaction.[91] However, a simple two-step irreversible M-M rate law (Equation 6.1) will again be assumed for this enzyme.

PPOX utilises an FAD (flavin adenine dinucleotide) cofactor which transfers electrons from the substrate to the electron acceptor oxygen.[94, 95] The K_M value of PPOX for the FAD cofactor, if found, could be incorporated into future versions of the 'Stage 1' model.

The parameters found from the literature for PPOX are presented in Table 6.7. The K_M values for protoporphyrinogen IX of this enzyme range from 0.85-9.75 (Table 6.7). Again this variation is potentially due to different sources, techniques and pH. Values from Shepherd and Dailey[409] were used for the 'Stage 1' model in Chapter 7.

Table 6.7 K_M and V_{max} Parameters Found for PPOX.

Organism	Location	V_{max} (µmol/l*min)	K_M (µM)	pH	°C	Reference
A. tuberculosis	—	—	1.12	7.4	37	410
Human	Plasmid	—	0.85	8.2	37	411
Human	Placenta	—	1.7	8.1	37	412
Human	Plasmid	3.8	3.8 (app)	8.0	37	409
Human	Erythrocyte	—	9.78	8.6	37	413
Human	Plasmid	—	2.33	7.4	37	414

K_M and V_{max} parameters found for protoporphyrinogen oxidase (PPOX); (app) indicates an apparent parameter.

6.2.2.8 Ferrochelatase (FECH)

$$protoporphyrin\ IX + Fe^{2+} \rightarrow protoheme + 2H^{+}$$

FECH (EC 4.99.1.1), the final enzyme of the heme biosynthetic pathway, catalyses the insertion of the ferrous form of iron into protoporphyrin IX forming heme. This enzyme is located in the mitochondrial inner membrane and is a dimer with each subunit consisting of similar domains.

FECH requires both iron and copper for activity and utilizes an iron-sulfur cluster as a cofactor. Copper stimulates the activity of FECH and decreases its K_M for iron although the exact mechanism for this is unknown. Ferrous iron is utilised as a substrate and inserted into protoporphyrin IX to form protoheme.

It has also been suggested that PLP may act as a factor or non-essential activator of FECH although this has not been confirmed.[96, 97] The potential mechanism and parameters for the involvement of PLP as an activator of FECH requires further investigation.

Table 6.8 V_{max} and K_M Parameters Found for FECH.

Organism	Location	V_{max} (µmol/l*min)	K_M PPIX (µM)	K_M Fe^{2+} (µM)	K_M Zn^{2+} (µM)	pH	°C	Reference
Mouse	Plasmid	—	15.3	11.1	—	8.1	37	415
Mouse	Plasmid	—	1.4	1.9	—	8.1	30	416
Human	Plasmid	6.6	9	9.3	—	8.1	25	417
Human	Erythrocytes	—	1.49	—	8.33	8.6	37	413
Human	Plasmid	—	16.8	10	—	8.1	37	415

K_M and V_{max} parameters found for ferrochelatase (FECH).

The parameter values from human sources reported K_M values of FECH and Fe^{2+} as close between references (9.3 and 10 μM), but the reported K_M for PPIX in humans varies significantly (from 1.49 - 16.8) (Table 6.8). For the 'Stage 1' model, data from Sellers et al.,[417] is used as this study reported a V_{max} value that is compatible with the model.

6.3 Summary of Chapter 6

In this chapter, the K_M and V_{max} values for heme biosynthetic pathway enzymes found from an exhausted literature review are reported. A table of all of the values used in the simple model can be found in Chapter 7 (Table 7.2). The complexity of heme biosynthesis is highlighted by the complex kinetic mechanisms of many of the enzymes discussed in this chapter.

The rate limiting enzyme ALAS has feedback inhibition and regulation at a number of levels, the kinetic parameters of which are not fully known. The combination of two of the same substrate (ALA) to form one product (PBG) by ALAD suggests there may be two different binding affinities; one for binding of the first ALA and one for binding of the second ALA to the enzyme already complexed with one ALA. However only one K_M value was found measured in the literature search and the simple irreversible M-M kinetics (Equation 6.1) was assumed.

PBGD is arguably the most complex enzyme of heme biosynthesis with the utilisation of two of its substrates bound together as a dipyrromethane cofactor (PBG-PBG) followed by the stepwise addition of four more of the same substrate. Again, only one

K_M value was found to represent this reaction, and Equation 6.1 is used to represent this reaction; this is a simplification of the kinetics involved.

The conversion of uroporphyrinogen III (or I) by UROS to coproporphyrinogen III (or I) is not a single-step reaction and occurs via intermediates which are also capable of spontaneous oxidation into porphyrins. While the K_M values of these intermediates are recorded in the literature,[402] this reaction will be treated as a single step reaction in the 'Stage 1' model of heme biosynthesis (Chapter 7) to provide a level of consistency with the other reaction parameters used in the model.

Although many of the parameters required to model the heme biosynthetic pathway have been found, there are still gaps in the parameter values needed for the creation of a comprehensive heme biosynthetic pathway model. More affinity constants (K_M values) were found recorded than V_{max} values. For consistency, all kinetic parameters chosen and used in the model described in Chapter 7, are from human sources with pH and temperature variables taken into account. Also, as V_{max} values are often found reported in different units, most of the values from the literature needed to be converted into common units (μmol/min*l) that could be utilised in the model described in the following chapter. If there was not enough information to report a usable value for an enzyme, it was not included in the tables in this chapter.

It is difficult to get accurate values that correlate well for every enzyme, due to the many variables and different sources of information resulting from a literature search of enzyme parameters. The different techniques, temperatures, and pH used to measure enzyme kinetic parameters by individual authors will result in some limitations to the accuracy of a system model using parameters from a literature

review. In addition, enzyme kinetic parameters derived from different sources (e.g., erythrocytes or hepatocytes) may have different parameter values. The parameters selected for use in the 'Stage 1' model were chosen based on a number of criteria designed to provide a level of consistency between the different enzymes whenever possible; parameters chosen were taken from the same species; recorded in the same units; and measured under similar pH and temperature conditions.

Other parameters that will be required to create more complicated future models of the heme biosynthetic pathway include feedback inhibition parameters, transport parameters between the cytosol and mitochondria as well as parameters for the reverse reactions of each enzyme in order to develop a model that is reversible and more relevant to *in vivo* conditions. The framework for the building of a complete functional computational model of heme biosynthesis will be presented in Chapter 7.

CHAPTER 7. 'STAGE 1' MODEL

7.1 Computational Modeling - COPASI

Biochemical modeling allows investigations of biological pathways through parameterization of individual reactions that are linked together through common substrates and products.[368] This allows for more holistic investigations into a process. Computational models are generally made up of ordinary differential equations (ODEs) that represent the rate of changes of the concentrations of chemical species within a system.[365, 368, 418] For the purposes of systems biology it has been suggested that it is sufficient to represent each enzyme-catalysed reaction as a single step and assign to it an appropriate integrated rate law.[368] For this reason, it has been proposed that rate laws for computational models can use generic rate laws to calculate fluxes through systems.[419] This has been termed "convenience kinetics", and is usually based on a generalised form of M-M kinetics. However, mechanistic details may affect the dynamics of systems, especially when there is inhibition or regulation of an enzyme involved.[368] Therefore, when simple kinetics are assumed, it must be remembered that there will be limitations to how accurately a model can represent a real system.

COPASI (COmplex PAthway SImulator) is an open source biochemical network modelling and simulation software package.[364] It is a stand-alone program that allows modelling of a system as well as investigations into the system using built in kinetic software. COPASI uses ODEs to represent the changes of chemical species in a system and was created for the simulation and analysis of biochemical networks. To date, no comprehensive model of heme biosynthesis has been created. In this chapter the creation of a simple model of heme biosynthesis based on published data of

individual enzymatic reactions is described. COPASI is the software used here to develop an initial heme biosynthetic pathway model; because of the models preliminary nature it is named: 'Stage 1'.

7.2 Stage 1 - Building a Simple Functional Model

7.2.1 Introduction

A simple and functional model of heme biosynthesis was created. Each step of the heme biosynthetic pathway was entered into a new COPASI model and parameterized primarily using the data reported in Chapter 6. The first step in the model is the ALAS reaction of succinyl-CoA and glycine to produce ALA (Table 7.1). This is followed by the other 7 enzymatic steps of the heme biosynthetic pathway. As heme is distributed, utilised, and degraded *in vivo,* there is a final ninth step in the model representing the flux into heme metabolism that occurs *in vivo*. This is represented as a single step. The assumptions and theory of the creation of this model will be discussed below.

7.2.2 'Stage 1' Model

For this initial model of the heme biosynthetic pathway a few arbitrary settings were assumed. For ease of calculations, all units of time were set to minutes, quantity units to μmoles, and volume units to liters. Only one compartment was opened in the model. In COPASI, compartments allow for representation of different biochemical locations (e.g. mitochondria and cytosol). As the parameters for transport between the cytosol and mitochondria were unknown, only one compartment was used in the model.

Each step of the heme biosynthetic pathway was assigned a reaction and rate law (Table 7.1). Cofactors and side products (i.e. PLP, CO_2, H_2O) were ignored for simplicity in this first functioning model. Other than ALAS and FECH all enzymatic reaction rate laws were set as irreversible and represented by M-M irreversible rate laws (Equation 6.1). As the ALAS and FECH catalyzed reactions utilize two different substrates with known K_M values, they were accordingly set as Bi irreversible (Equation 2). The final step of the pathway, heme utilisation, was represented as a non-enzymatic single step with a mass action rate law. This final step of the pathway allows the model to reach a steady state, as in normal *in vivo* situations, heme synthesis is in perfect balance with heme metabolism and does not accumulate in excess. For simplicity, feedback inhibition was not included in this model. Rate laws and reactions for each step in the model are summarised in Table 7.1.

The specific kinetic parameters for each of the rate laws were selected from the literature review as described in Chapter 6 and the parameters are summarised in Table 7.2. All enzymatic parameters chosen were either from human subjects or from human protein expressed in *E. coli.* For the final step of the model, heme utilisation, the rate was chosen through a trial and error process to find a rate that would balance heme synthesis with heme metabolism and allow the model to reach a steady state.

Table 7.1 Reactions and rate laws for ‘Stage 1’ model.

1	1 ALAS	glycine + succinyl-CoA -> ALA	Bi (irreversible)	2.93933e-06
2	2 ALAD	2 * ALA -> PBG	Rate Law for 3 PBGD	1.46966e-06
3	3 PBGD	4 * PBG -> HMB	Rate Law for 3 PBGD	3.67416e-07
4	4 UROS	HMB -> uroporphyrinogenIII	Henri-Michaelis-Menten (irreversible)	3.67416e-07
5	5 UROD	uroporphyrinogenIII -> coproporphyrinogenIII	Henri-Michaelis-Menten (irreversible)	3.67416e-07
6	6 CPOX	coproporphyrinogenIII -> protoporphyrinogenIX	Henri-Michaelis-Menten (irreversible)	3.67416e-07
7	7 PPOX	protoporphyrinogenIX -> protoporphyrinIX	Henri-Michaelis-Menten (irreversible)	3.67416e-07
8	8 FECH	protoporphyrinIX + Fe2+ -> protoheme	Bi (irreversible)	3.67416e-07
9	Heme utilisation	protoheme ->	Mass action (irreversible)	3.67416e-07

Table recording the enzymes, reactions, rate laws and flux of reactions in the ‘Stage 1’ model.

Table 7.2 'Stage 1' parameters.

Enzyme	Rate Law	K_M (µmol/l)	V_{max} (µmol/(l*min)	Reference
ALAS	Bi	Glycine: 9,750 Succinyl-CoA: 43.9	5.09	78, 378
ALAD	M-M	270	1.26	385
PBGD	M-M	48	0.105	389
UROS	M-M	0.15	0.00023	397
UROD	M-M	0.4	0.004176	402
CPOX	M-M	0.291	0.264 x 10-9	406
PPOX	M-M	3.8	0.285	409
FECH	Bi	PPIX: 9 Fe2+: 9.3	6.6	417
HemeD	Mass action	0.003 (1/min)		n/a

Rate law and K_M and V_{max} parameters used for each step of the heme biosynthetic pathway in the 'Stage 1' model of heme biosynthesis.

The concentrations of the initial precursors glycine, succinyl-CoA and Fe^{2+} were set as 'fixed'; that is set at a constant concentration that is not altered by changes elsewhere in the pathway. As these three substrates feed into the heme biosynthetic pathway this was done to reflect constant flux into the heme biosynthetic pathway. All other metabolite (species) concentrations of intermediates of the heme biosynthetic pathway were set to 'reactions' in COPASI which allows the concentrations of these species to be altered by flux of the heme biosynthetic pathway.

The initial concentrations of the precursors glycine and succinyl-CoA were set at the fixed concentration of 0.5 µM, an arbitrary physiologically reasonable concentration, to allow constant flux into the model. This is summarized in Table 7.3. As adequate concentrations of Fe^{2+} proved important in the model at the final enzymatic step of the pathway, Fe^{2+} concentration was set at the high concentration of 100 µM. Significantly lowering concentration of Fe^{2+} resulted in lowered output of heme and

higher concentrations of protoporphyrin IX. All other porphyrin precursors were set initially at 0 μM in order to measure flux through the heme biosynthetic pathway as concentrations *in vivo* are difficult to quantify (Table 7.3).

Table 7.3 Species, reactions, and intitial concentrations.

#	Name	Compartment	Type	Initial Concentration (μmol/l)
1	ALA	compartment	reactions	0
2	glycine	compartment	fixed	0.5
3	succinyl-CoA	compartment	fixed	0.5
4	PBG	compartment	reactions	0
5	HMB	compartment	reactions	0
6	uroporphyrinogenIII	compartment	reactions	0
7	coproporphyrinogenIII	compartment	reactions	0
8	protoporphyrinogenIX	compartment	reactions	0
9	protoporphyrinIX	compartment	reactions	0
10	Fe2+	compartment	fixed	100
11	protoheme	compartment	reactions	0

Summary of species and initial concentrations of the 'Stage 1' model of the heme biosynthetic pathway.

7.2.3 Steady State

As previously mentioned, a steady state is reached when the concentrations of the intermediates of a pathway remain constant because the rates of formation have come to be in exact balance with their rate of utilization. In order to reach a steady state, the end product of the pathway, in this case protoheme, was assumed to be utilised (Table 7.1). If this assumption is removed from the model, heme concentration rises continuously and a steady state is not reached (Figure 7.2).

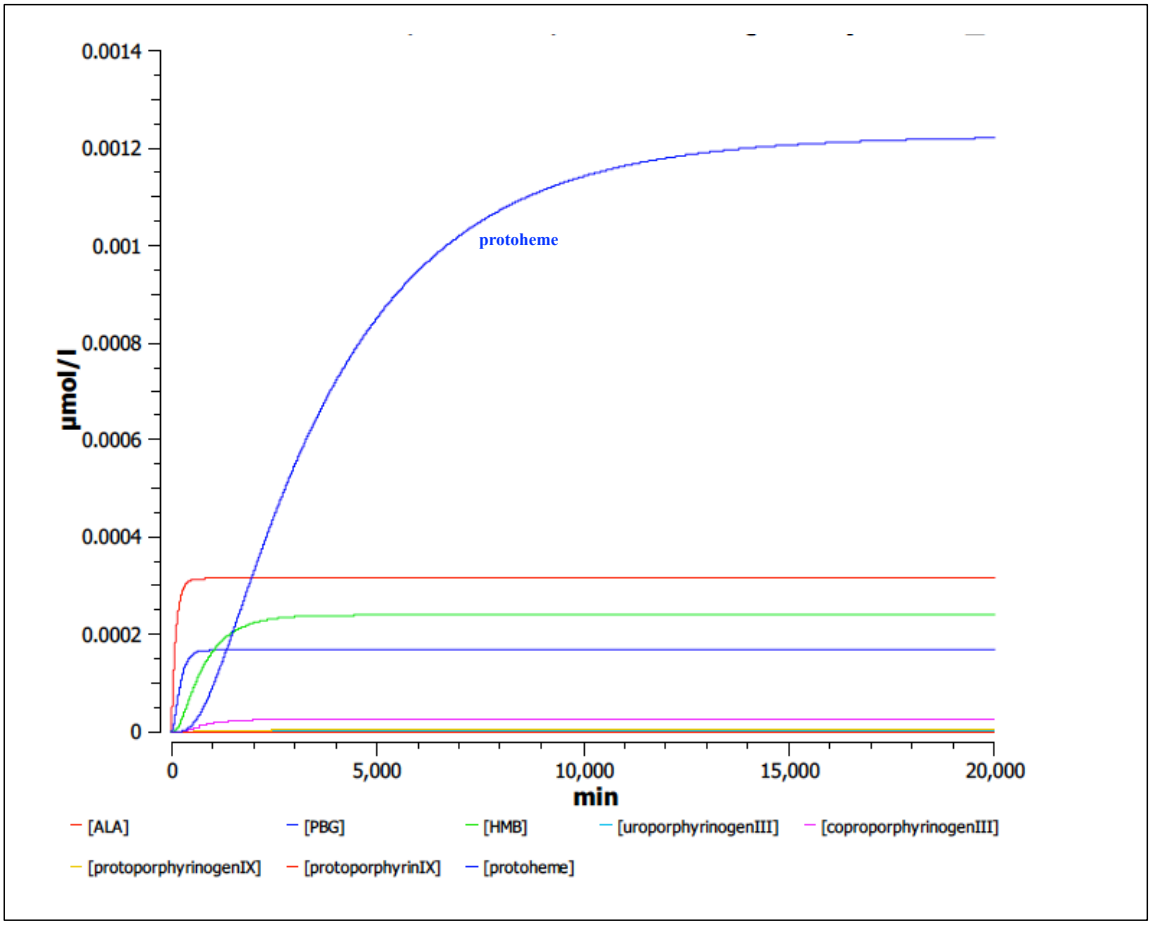

Figure 7.1 Flux of metabolites of 'Stage 1' model.

Model has reached steady state due to the 9th step of the 'Stage 1' model: heme utilization.

One of the useful features of COPASI is the way it summarises information. The steady state of the 'Stage 1' model is summarized in Table 7.4. This table shows the concentrations of each metabolite (species) at steady state. As we would expect to see *in vivo*, protoheme has the highest concentration and there are very low levels of the porphyrinogens. However, we would expect to see much lower calculated concentrations of HMB than the porphyrinogens as HMB is highly unstable and never accumulates *in vivo*. In a future models the non-enzymatic cyclisation of HMB to uroporphyrinogen I can be added as a side pathway (see Figure 2.1); this may resolve the discrepancy and deliver lower calculated concentrations of HMB in the

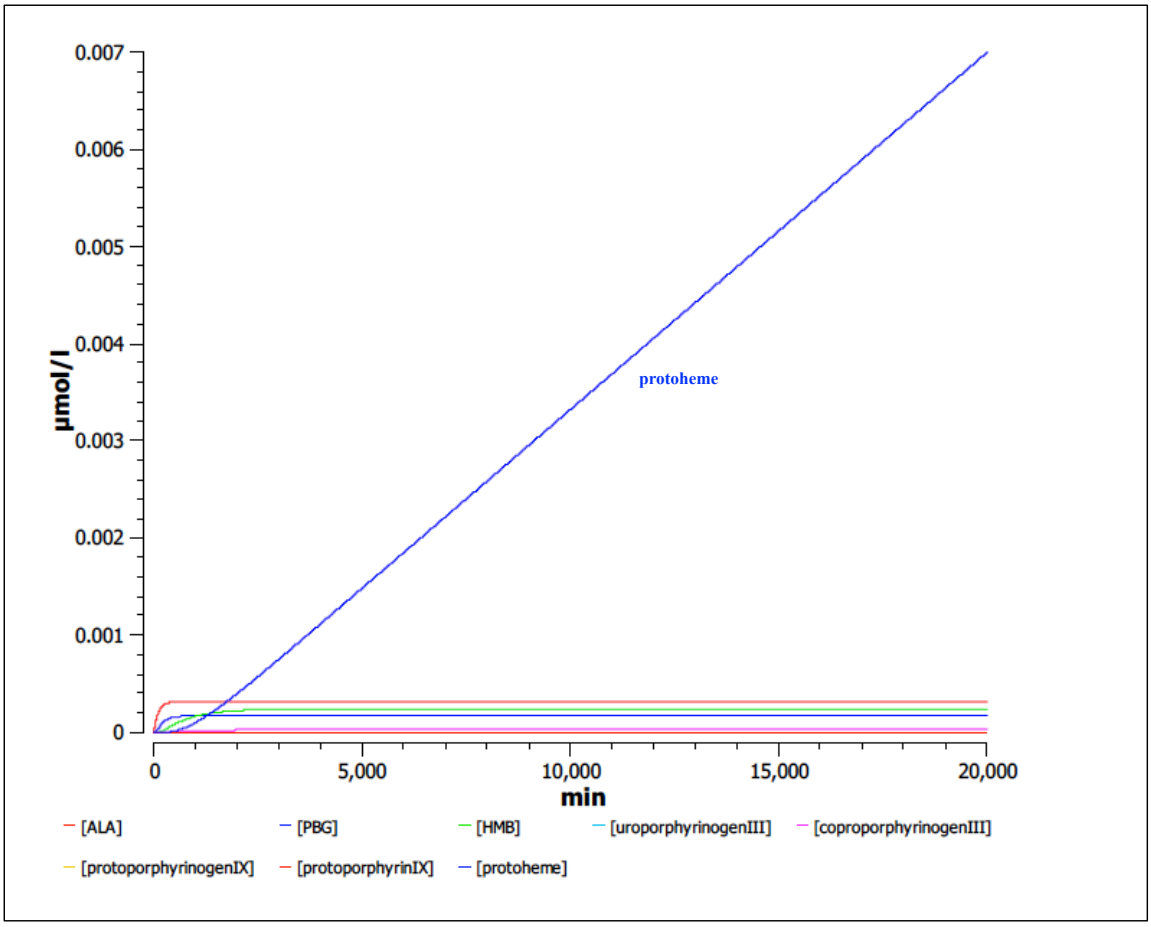

Figure 7.2 Flux of metabolites of 'Stage 1' model without heme utilization.

When heme utilization is removed, protoheme continuously rises and the model does not reach a steady state.

model. In conclusion, the model did successfully reached a steady state which is critical before performing investigations into the effect of changing enzyme parameters of the model to investigate the porphyrias. Concentrations of metabolites at steady state, rate of metabolism and time it took to reach steady state are summarised in Table 7.4 and reflect the flux of the pathway shown in Figure 7.1.

Table 7.4 Calculated Steady State Paramaters of 'Stage 1' Model.

	Name	Type	Concentration (µmol/l)	Rate (µmol/(l*min))	Transition Time (min)
1	ALA	reactions	0.000314928	-4.05405e-22	107.143
2	PBG	reactions	0.000167962	2.02702e-22	114.286
3	HMB	reactions	0.000240002	5.06756e-23	653.217
4	uroporphyrinogenIII	reactions	2.28743e-06	-5.06756e-23	6.22572
5	coproporphyrinogenIII	reactions	2.56052e-05	0	69.69
6	protoporphyrinogenIX	reactions	4.89888e-06	-5.06756e-23	13.3334
7	protoporphyrinIX	reactions	5.47617e-07	5.06756e-23	1.49045
8	protoheme	reactions	0.00122472	0	3333.33

Steady state calculations of the 'Stage 1' model are summarized including: concentration at steady state, rate, and transition time.

7.2.4 Stoichiometry Analysis

As discussed in previous chapters, it takes eight molecules of succinyl-coA and eight molecules of glycine at the beginning of the pathway to result in one molecule of heme. This relates to the stoichiometry of the model. Stoichiometric coefficients report the proportions between substrate and product molecules involved in a process. In Table 7.5, the stoichiometry of species in relation to individual reactions is shown. Positive values are shown in green and negative values are shown in red, this represents the creation and utilization of metabolites. For example, we can see in column ALA, row (2 ALAD), that 2 molecules of ALA are being utilised in the ALAD reaction. This describes the stoichiometry known to occur *in vivo*. The proportion of species utilised in the overall pathway is shown in Table 7.6.

Table 7.5 Stoichiometry analysis of reactions.

	(1 ALAS)	(2 ALAD)	(3 PBGD)	(4 UROS)	(5 UROD)	(6 CPOX)	(7 PPOX)	(8 FECH)	(Heme degredation)
PBG	0	1	-4	0	0	0	0	0	0
ALA	1	-2	0	0	0	0	0	0	0
uroporphyrinogenIII	0	0	0	1	-1	0	0	0	0
protoporphyrinogenIX	0	0	0	0	0	1	-1	0	0
protoheme	0	0	0	0	0	0	0	1	-1
protoporphyrinIX	0	0	0	0	0	0	1	-1	0
coproporphyrinogenIII	0	0	0	0	1	-1	0	0	0
HMB	0	0	1	-1	0	0	0	0	0

Columns represent reactions and rows represent species altered by the reaction. Positive values are green and negative values are red.

Table 7.6 Stoichiometry analysis of overal pathway.

List | Net Reactions | EFM vs Reactions | EFM vs Species

#	ALA	glycine	succinyl-CoA	PBG	HMB	uroporphyrinogenIII	coproporphyrinogenIII	protoporphyrinogenIX	protoporphyrinIX	Fe2+	protoheme
1	-8 | +8	-8 | +0	-8 | +0	-4 | +4	-1 | +1	-1 | +1	-1 | +1	-1 | +1	-1 | +1	-1 | +0	-1 | +1

Representation of the stoichiometry of the species (metabolites) in the overall heme biosynthetic pathway.

7.2.7 Metabolic Control Analysis

Metabolic control analysis is a method of quantifying how much the rates of individual reactions affect the concentrations and fluxes of the steady state. The rate-limiting step of a pathway is defined as the slowest step. However, in a metabolic steady state all of the individual reactions are going at the same rate. Therefore metabolic control analysis is relevant to measure how a change in an individual reaction will affect the steady state. COPASI provides the software technology to easily perform metabolic control analysis through the creation of reports of elasticities, flux control coefficients and concentration control coefficients which will each be described.

Elasticity coefficients reflect the relationship between the change of a reaction rate with the change of the concentration of a chemical species.[368] COPASI calculates the elasticities of all the reactions with respect to all of the species in a model. A positive value denotes metabolites that stimulate the rate of a reaction and negative values denote metabolites that inhibit a reaction. The elasticity coefficients simulated by COPASI for the 'Stage 1' model are shown in Table 7.7. It should be noted that the elasticity table created by COPASI does not list the metabolite concentration columns in the order they appear in the heme biosynthetic pathway. All values shown in Table 7.7 are positive values and represent the affect of a substrate(s) on its reaction(s). This is to be expected with the simple 'Stage 1' model as no feedback inhibition is included. Future models, that include the feedback inhibition of protoheme on ALAS as well as of porphyrinogens on PBGD will produce negative elasticity coefficients at the respective enzymatic steps that are inhibited. Glycine, succinyl-CoA, and Fe^{2+} are

set at ‘fixed’ concentrations in the model and are not shown in Table 7.7 as their concentrations do not change.

Flux control coefficients, unlike elasticities, are global properties that depend on the model as a whole.[368] They record the extent of change in the flux of a reaction when another reaction is made to go faster or slower. In other words, they represent how much the steady state flux of a step in a metabolic pathway will change with altering the enzyme concentration of another step. If a flux coefficient is low, changing the concentration of that enzyme will have less effect on the pathway. Again, in Table 7.8, negative values reflect negative effect while positive values reflect a positive effect. The metabolic control analysis of the ‘Stage 1’ model by COPASI generated a table of flux control coefficients for the model with columns representing cause and rows representing effect (Table 7.8). Interestingly, PPOX and FECH appeared to have minor effects on the flux of later steps of the pathway, whereas CPOX significantly affected the flux through UROD, and ALAD affected flux of PBGD. However, the most significant result of Table 7.8 is the fact that ALAS had an effect on the flux of every step of the pathway, which is known to be the case *in vivo*. This supports the reported fact that ALAS is effectively the rate-limiting enzyme of the heme biosynthetic pathway and supports the validity of the ‘Stage 1’ model as a simple representative of heme biosynthesis.

Concentration control coefficients are similar to flux control coefficients, yet they describe changes in steady-state concentrations of species rather than flux through a step in response to changes of specific reaction rates.[368] Flux control coefficients of the ‘Stage 1’ model are shown in Table 7.9 with columns representing cause and rows representing effect. Again, it should be noted that in Table 7.9 COPASI does not

Table 7.7 Metabolic control analysis: Elasticities.

	PBG	ALA	uroporphyrinogenIII	protoporphyrinogenIX	protoheme	protoporphyrinIX	coproporphyrinogenIII	HMB
(1 ALAS)	0	0	0	0	0	0	0	0
(2 ALAD)	0	0.999999	0	0	0	0	0	0
(3 PBGD)	0.999996	0	0	0	0	0	0	0
(4 UROS)	0	0	0	0	0	0	0	0.998403
(5 UROD)	0	0	0.999994	0	0	0	0	0
(6 CPOX)	0	0	0	0	0	0	0.999912	0
(7 PPOX)	0	0	0	0.999999	0	0	0	0
(8 FECH)	0	0	0	0	0	1	0	0
(Heme degredation)	0	0	0	0	1	0	0	0

(rows = effect; columns = cause)

Table 7.8 Flux control coefficients.

	(1 ALAS)	(2 ALAD)	(3 PBGD)	(4 UROS)	(5 UROD)	(6 CPOX)	(7 PPOX)	(8 FECH)	(Heme utilisation)
(1 ALAS)	1	0	0	0	0	0	0	0	0
(2 ALAD)	1	0	0	0	0	0	0	0	0
(3 PBGD)	1	-1.24344e-16	0	0	0	0	0	0	0
(4 UROS)	1	0	0	0	0	0	-1.73763e-16	1.73763e-16	0
(5 UROD)	1	0	0	0	0	-1.42662e-16	-1.73763e-16	1.73763e-16	0
(6 CPOX)	1	0	0	0	0	0	-1.73763e-16	1.73763e-16	0
(7 PPOX)	1	0	0	0	0	0	0	1.73763e-16	0
(8 FECH)	1	0	0	0	0	0	-1.48978e-16	2.22045e-16	0
(Heme utilisation)	1	0	0	0	0	0	-2.72848e-16	2.72848e-16	0

(rows = effect; columns = cause)

Table 7.9 Concentration control coefficients.

	(1 ALAS)	(2 ALAD)	(3 PBGD)	(4 UROS)	(5 UROD)	(6 CPOX)	(7 PPOX)	(8 FECH)	(Heme utilisation)
PBG	1	-1.24345e-16	-1	0	0	0	0	0	0
ALA	1	-1	0	0	0	0	0	0	0
uroporphyrinogenIII	1.00001	0	0	0	-1.00001	-1.42663e-16	-1.73764e-16	1.73764e-16	0
protoporphyrinogenIX	1	0	0	0	0	0	-1	1.73764e-16	0
protoheme	1	0	0	0	0	0	-2.72848e-16	2.72848e-16	-1
protoporphyrinIX	1	0	0	0	0	0	-1.48978e-16	-1	0
coproporphyrinogenIII	1.00009	0	0	0	0	-1.00009	-1.73779e-16	1.73779e-16	0
HMB	1.0016	0	0	-1.0016	0	0	-1.74041e-16	1.74041e-16	0

(rows = effect, columns = cause) - green = positive value, red = negative value

generate the species column in the order they appear in the heme pathway. As expected, each reaction resulted in a negative concentration coefficient of its substrate (highlighted in red). Because the flux of succinyl-CoA and glycine as well as Fe^{2+} are not affected by the model, they are not reported in Table 7.9. However, the 'Stage 1' model, does report a positive concentration control coefficient of ALAS for every variable substrate of the model which is also in support of the rate-limiting role of this enzyme in heme biosynthesis.

7.2.8 Summary of 'Stage 1' Model

"Everything should be made as simple as possible but not too simple" - Albert Einstein.

In summary, a model of the heme biosynthetic pathway has been created with the use of a number of simplifying assumptions. The model successfully reached a steady state and flux through the pathway resembles that which occurs *in vivo*. Having achieved this, the 'Stage 1' model was investigated to discern what happens to the flux of the modeled pathway with a change in one of the parameters. The outcome of changing individual parameters allows strengths and weaknesses of the model for future iterations to be evaluated.

7.3 Stage 2 - Investigations into AIP

7.3.1 Modeling AIP

The ability of the 'Stage 1' model to describe the biochemical effects of a porphyria was undertaken for AIP. This porphyria was chosen because K_M and V_{max} parameters for this PBGD have been recorded in both normal and AIP subjects (Table 6.3). As

alterations of PBGD activity appear to be the underlying factor in the acute porphyrias (with the exception of ALAD-P), this enzyme is relevant to future investigations into the vitamin B6-related hypothesis for the biochemistry of acute attacks. Changing activities of CPOX or PPOX (mutations of which cause HCP and VP respectively) would not represent the biochemistry of acute porphyria in the simple 'Stage 1' model as the relevant feedback inhibition has not been incorporated.[93] Thus PBGD presented itself as a good choice for the initial modeling of a simplistic porphyric state.

The K_M and V_{max} values of PBGD were changed to 1579 μM and 0.051 μmol/(l*min) respectively, values measured in AIP patients.[389] These values were from the same source as the 'normal' parameters of PBGD used in the 'Stage 1' model. All other parameters values were kept the same as in the 'Stage 1' model.

7.3.2 Steady State and Metabolic Control Analysis

This new 'AIP' model showed increased concentration of PBG and decreased rate of heme formation at steady state (Figure 7.3). The 'AIP' model took longer to reach a steady state (Table 7.10) than in the previous 'Stage 1' model (Table 7.4). The 'AIP' model reported PBG concentration at steady state to be considerably elevated (Figure 7.3). This is expected with diminished activity of PBGD and occurs in AIP patients.[301-304] The transition time to reach a steady state concentration of PBG was also considerably increased (Table 7.10) in comparison with the initial model (Table 7.4). Although this is a step in the right direction for modelling the biochemistry of AIP, PBG levels alone were elevated, whereas in AIP both ALA and PBG levels are

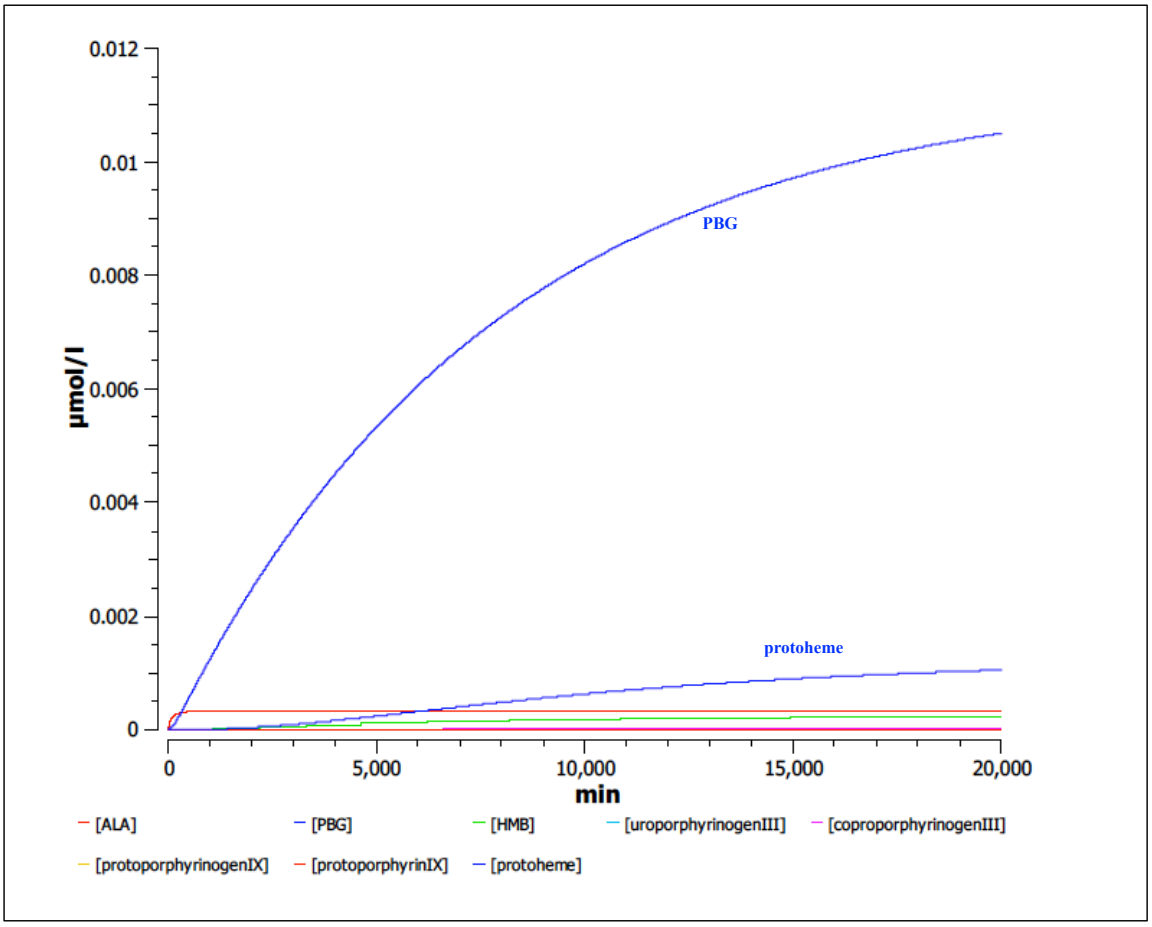

Figure 7.3 Model of the flux through the 'AIP' version of the 'Stage 1' model.

elevated (Table 3.1). This is likely due to the irreversible rate laws assumed for the enzymatic reactions of the model, whereas in physiological conditions there would be some flux in both directions at each step. This could also be due to the lack of either representation of feedback inhibition of ALAS by heme or the ALAS up-regulation when heme levels are diminished.

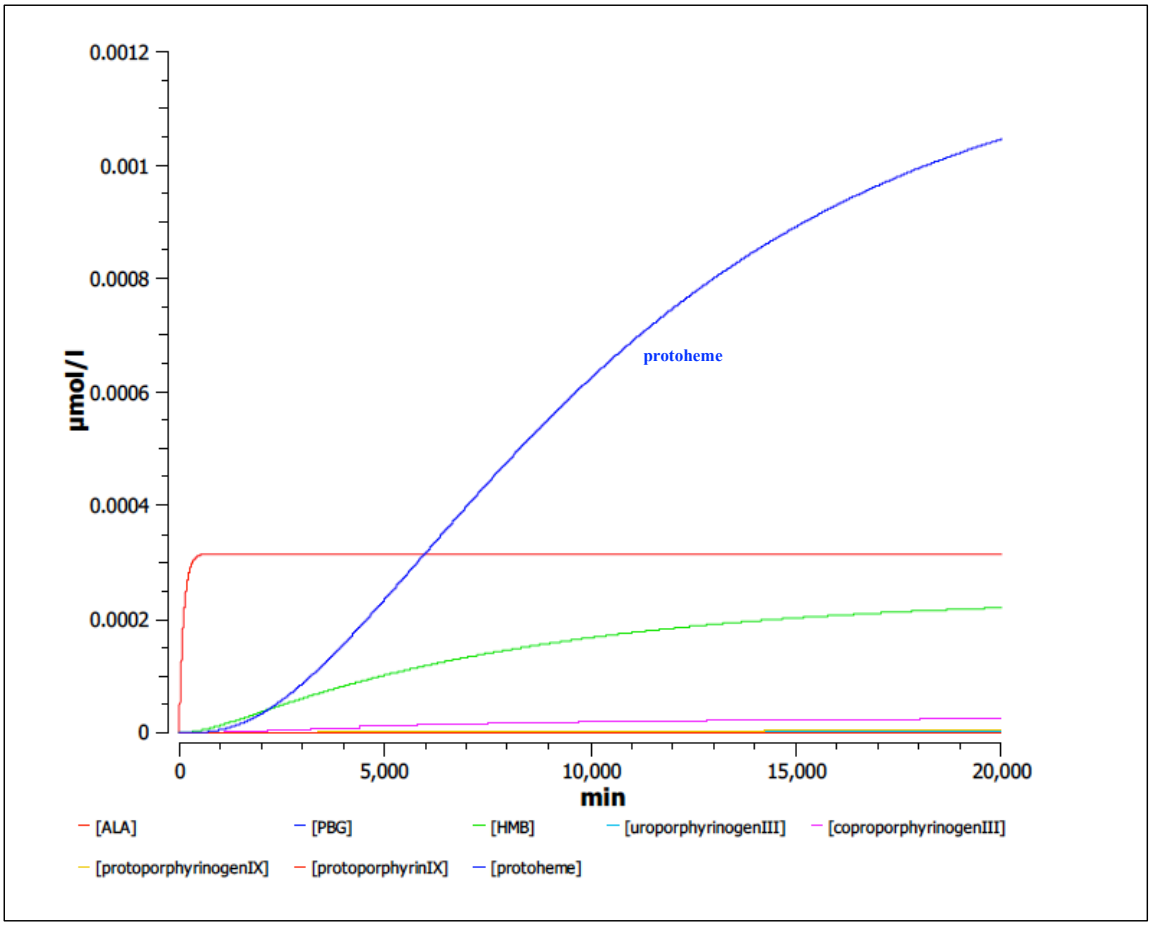

Figure 7.4 'AIP' Model With PBG Levels Removed.

Model of the flux through the 'AIP' version of the 'Stage 1' model with PBG levels removed. This can thus be compared with Figure 7.1. at a similar scale.

Table 7.10 Steady State of the "AIP" Model.

	Name	Type	Concentration (µmol/l)	Rate (µmol/(l*min))	Transition Time (min)
1	ALA	reactions	0.000314928	4.05405e-22	107.143
2	PBG	reactions	0.0113756	-2.02702e-22	7740.25
3	HMB	reactions	0.000240002	-5.06756e-23	653.217
4	uroporphyrinogenIII	reactions	2.28743e-06	5.06756e-23	6.22572
5	coproporphyrinogenIII	reactions	2.56052e-05	0	69.69
6	protoporphyrinogenIX	reactions	4.89888e-06	1.52027e-22	13.3334
7	protoporphyrinIX	reactions	5.47617e-07	-1.52027e-22	1.49045
8	protoheme	reactions	0.00122472	0	3333.33

Steady state calculations of the 'AIP' model are summarized including: concentration at steady state, rate, and transition time.

It is worth noting that when the 'AIP' model is compared with the initial 'Stage 1' model of heme synthesis (Figure 7.1), without the elevated PBG and at the same scale, heme is still being formed at similar levels although the rate of the initial heme formation is slower (Figure 7.4). This shows that the rate of protoheme formation is slower in the AIP model yet it still reaches the same concentration level at steady state (0.00122472) (Tables 7.4 and 7.10).

7.3.3 Summary of AIP Model

The fact that changing the parameters of one of the enzymes associated with porphyria resulted in changes of flux through the heme biosynthetic pathway shows promise for future investigations. However, the 'AIP' model also reflects some of the limitations of this preliminary model as it does not completely represent AIP biochemistry. These results are useful as they highlight changes that are needed to produce future heme biosynthesis models that will more accurately model porphyria biochemistry.

7.4 Stage 3 - Future work

Although this model utilises a number of assumptions, it is a useful starting point for the building of more complex models that can be used to investigate the heme biosynthetic pathway and the porphyrias. The fact that the model has reached a steady state and can measure flux through the pathway, as well as represent some of the biochemical changes associated with porphyria, shows promise for future work. A number of changes will be presented that are needed before the model can represent a physiologically relevant situation with some accuracy.

7.4.1 Rate Laws

Perhaps the first and most important change to the model would be the incorporation of reversible rate laws. The fact that the "AIP" model did not completely reflect actual AIP metabolite flux, may be due to the fact that simple irreversible reactions were assumed. In order to include these rate laws, the parameters for both the forward and reverse reactions would need to be measured experimentally. The reversible M-M rate law is (Equation 6.4):

Equation 6.4:

$$A + B \rightarrow P$$

$$v = \frac{V_{max}for \cdot K_M p}{V_{max}rev \cdot K_M S}$$

To be more biologically relevant, new rate laws should include a direct parameter for enzyme concentration. As enzyme concentration is not directly written into Equation 6.4, perhaps the parameter kcat (turnover number) would be a better parameter to use rather than V_{max}; V_{max} is defined as the product of kcat and enzyme concentration. Thus Equation 6.4 would be written as:

Equation 6.5:

$$v = \frac{E \cdot kcatfor \cdot K_M p}{E \cdot kcatrev \cdot K_M S}$$

With an enzyme concentration value incorporated into the model, effects of changes in gene expression and hence enzyme concentrations could also be altered in order to investigate porphyria. Some porphyria mutations appear to result in lowered enzyme concentration rather than altered enzyme binding (K_M) or V_{max}. Suggested "convenience kinetics" reversible rate laws for computational modelling of biological

systems have been reported.[419] It would also be useful in the development of a heme biosynthetic pathway model if separate K_M values can be found for the different levels of substrate binding in the ALAD and PBGD reactions. These parameters could then be integrated into future rate laws although this is not essential for the purpose of computational models of whole systems.

Transport parameters were not included in the model. With further development of the model, these parameters would ideally be included. COPASI is capable of representing multiple 'compartments' within a system. Heme biosynthesis occurs in both the mitochondria and the cytosol with transport of heme precursors between these two 'compartments'. However, as values for transport parameters were not found from the literature search, they were not incorporated into the 'Stage 1' model.

7.4.2 Regulation and Inhibition

As the feedback inhibition of ALAS by heme is important in the regulation of the pathway as well as being significant in the pathophysiology of the porphyrias, this must also be included in an upgraded version of the model. This would prove beneficial even if the mechanism of the first step of the pathway is represented by simple uncompetitive inhibition kinetics and a reasonable arbitrary value is inserted as the inhibition constant to mimic *in vivo* situation.

In addition, as HCP and VP present with acute symptoms due to inhibition of PBGD (the enzyme deficient in AIP) by coproporphyrinogens and protoporphyrinogen inhibition, parameters for these heme precursors should be incorporated into the rate law of PBGD. Any other known inhibitors and activators should also be incorporated, i.e. lipoic acid at UROD.

7.4.3 Branches in the Pathway

In future heme biosynthetic pathway models, pathway branches should also be included. HMB's ability to non-enzymatically cyclise into uroporphyrinogen I as well as the conversion of uroporphyrinogen I into coproporphyrinogen I by UROS should be included (Figure 2.1). It may be the lack of this side pathway that resulted in the higher than expected (although still low) concentrations of the unstable HMB in the 'Stage 1' model of the heme biosynthetic pathway. The parameters for the enzymatic reaction of porphyrinogen I isomers have been reported in the literature (Table 6.5).

Another side-pathway that may be worth including is porphyrinogens to porphyrins. Porphyrinogen precursors of heme are rapidly oxidised to porphyrins with a loss of six protons upon exposure to air. As it is the porphyrins rather than porphyrinogens that accumulate in the porphyrias, it may be worth including these rapid oxidation reactions in future models as well.

7.4.4 Cofactors

It has been suggested that: *"The distinction between substrates and coenzymes, useful though it may be in physiological studies, has no meaning in relation to enzyme mechanisms" – Cornish-Bowden.*[420]

Bearing this in mind, in the next stage of the model it would be useful to include cofactors into the rate laws and reactions. This would allow investigations of the pathways in relation to dependence and utilisation of the cofactors which we have previously suggested may have a significant affect in the pathophysiology of the porphyrias. This is essential to future investigations of the hypothesis presented in Chapter 5.

7.4.5 Pathway Expansion

In order to investigate the biochemistry of the acute attack in relation to micronutrient depletion (Chapter 5), interconnected biochemical pathways would also need to be parameterised. These pathways would include the TCA cycle, cofactor synthesis and metabolism, as well as some of the pathways that protoheme feeds into including the tryptophan/serotonin pathway, which has already been modeled.[357] This begins to get exceedingly complex and it would require methodical 'step by step' additions to the pathway, which would lead to a more complicated model further down the track.

7.4 Summary of Chapter 7

Although the model presented as 'Stage 1' is very simplistic, it presents the groundwork for the creation of more complex as well as more physiologically significant models of heme biosynthesis. *"Despite considerable advances in the topological analysis of metabolic networks, inadequate knowledge of the enzyme kinetic rate laws and their associated parameter values still hampers large-scale kinetic modelling" – Adiamah et al.*[421]

More knowledge of the kinetic rate laws and parameters of the heme biosynthetic pathway enzymes are required to successfully advance the model. However, there is undoubtably potential in utilising a future, more complex, model in carrying out "what-if" experiments relevant to porphyria. This could allow for breakthroughs into the understanding of the mechanism behind the acute porphyric attack as well as investigations with the focused on safer and more effective treatment regimes.

CHAPTER 8. CONCLUSION

8.1 Aims of Thesis

The aims at the start of this thesis were to:

1.) Review literature on effects of micronutrients in the porphyrias with the aim of developing a testable hypothesis that could aid future treatments.

2.) Develop a methodology suitable for testing the hypothesis.

8.2 Conclusion of Thesis

As seen throughout this thesis, the porphyrias are a diverse and complex family of disorders. This complexity is reflected in the fact that some patients with the underlying genetic defect(s) remain latent while others are symptomatic. This indicates there is at least one other factor required in addition to a genetic defect for the development of porphyria symptoms. A systematic review of the relationships between heme biosynthesis, the porphyrias, and micronutrients was undertaken. A component of this provided a much needed review of reported micronutrient treatments of the porphyrias while another component inspected possible mechanisms for the acute attack of porphyria.

Evidence of micronutrient deficiencies were found to be widely reported among the porphyrias (Table 4.2) as was successful micronutrient treatment (Table 4.3) albeit only in restricted locations. This adds credibility to the proposal that micronutrient deficiencies, in particular vitamin B6 (PLP) may play a role in the pathogenesis of both the acute and cutaneous porphyrias. Many of these reports were written in languages other than English. Controlled trials to determine the effectiveness of these

treatments as well as to determine optimal dosage and combinations of micronutrients would be beneficial to the porphyric population.

Through considerations of the heme biosynthetic pathway (as well as connected pathways), clinical presentation of the porphyrias, successful treatments of porphyria, and suggested mechanisms for the pathophysiology of the acute porphyric attack, a hypothesis was formed for the etiology of the manifestations of acute porphyria:

It is hypothesized that a PLP deficit is induced or exacerbated in the porphyrias by the up-regulated activity of the early steps of the heme biosynthetic pathway and that this PLP deficit and the subsequent cascade of biochemical insults resulting from this deficit is the precipitaiting factor required in addition to the genetic mutation for the manifestation of the symptomology of the acute porphyrias.

The 'biochemical insults' that could result from a vitamin B6 (PLP) deficiency are numerous and include additional micronutrient deficiencies, altered processes that require PLP-cofactors (e.g. tryptophan/serotonin pathways), and reduced GABA synthesis. The altered tryptophan/serotonin pathway could lead to higher levels of circulating serotonin with serotonergenic effects. Reduced synthesis of GABA, an important neurotransmitter, may result in lack of competition with ALA due to their similar structures resulting in increased ALA toxicity. Although hypotheses of *in vivo* biochemistry can be difficult to 'prove', further studies of PLP as a treatment of porphyria as well as measuring PLP levels in both symptomatic and asymptomatic porphyria patients could support or disprove this hypothesis. However, due to the

rarity and variability of porphyria, it is difficult to do human studies with statistically significant results.

In order to develop another mechanism to test this hypothesis, a novel new approach was taken through building a computational model of heme biosynthesis. This model was parameterised using kinetic data from a literature review which added to the reservoir of knowledge of the individual reactions of heme biosynthesis. Numeric modeling using the program COPASI was applied to create a simple model with a steady state that simulated a simple reflection of *in vivo* heme biosynthesis. However, changing the parameters of an enzyme to model the mutation found in AIP did not completely represent the biochemistry of AIP. This highlights limitations of the model and areas for future work. There are significant gaps in the literature available for kinetic parameters of heme biosynthesis, thus hindering model expansion. More information is currently available is required before a full capacity model can be built with the potential to investigate the hypotheses proposed in previous chapters.

Heme biosynthesis is a fascinating and complex process. The building of a comprehensive model of the heme biosynthetic pathway will allow testing of different hypotheses in an area where this was previously unable. Although the model presented in this thesis does not yet reflect a fully functioning representation of heme synthesis it provides a simple functioning model that lays the groundwork for future studies.

In conclusion, it has been hypothesized that a PLP (vitamin B6) deficiency may be the risk factor necessary in conjunction with the genetic defect in porphyria to trigger acute attacks. If this is the case, micronutrient therapy may provide a safe method for

the treatment and prophylaxis of the porphyrias. A computational model of the heme biosynthetic pathway has the potential to investigate hypotheses of the disordered biochemistry of the porphyrias as well as the aid the discovery of new methods of treatment and symptom control.

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GENERAL ABBREVIATIONS

A list of abbreviations used:

AIP	acute intermittent porphyria	NAD^+	nicotinamide adenine dinucleotide
ALA	aminolevulinate	NADP(H)	nicotinamide adenine dinucleotide phosphate
ALAD-P	aminolevulinate dehydratase deficiency porphyria	ODE	ordinary differential equation
ALAS	aminolevulinate synthase	PBG	porphobilinogen
BBB	blood brain barrier	PBGD	porphobilinogen deaminase
BRENDA	braunschweig enzyme database	PCT	porphyria cutanea tarda
CEP	congenital erythropoietic porphyria	PGC-1	proliferator-activated receptor coactivator 1
CNS	central nervous system	PLP	pyridoxal 5'phosphate
CoA	coenzyme A	PPOX	protoporphyrinogen oxidase
COPASI	complex pathway simulator	TCA	tricarboxylic
CPOX	coproporphyrinogen oxidase	THFA	tetrahydrofolic acid
EPP	erythropoietic protoporphyria	ThPP	thiamine pyrophosphate
FAD	flaven adenine dinucleotide	IU	international unit
GABA	γ-aminobutyric acid	UROD	uroporphyrinogen decarboxylase
HCP	hereditary coproporphyria	UROS	uroporphyrinogen synthase
HEP	hepatoerythropoietic porphyria	UV	ultraviolet
HIV	human immunodeficiency virus	VP	variegate porphyria
HMB	hydroxymethylbilane	XLPP	X-linked dominant protoporphyria
IV	intravenous	XLSA	X-linked sideroblastic anemia

Summary of the Heme Biosynthetic Pathway With a List of Abbreviations for the Metabolites, Enzymes, and Porphyrias Involved

Key Abbreviations	
ADP	ALAD deficiency porphyria
AIP	acute intermittent porphyria
ALAD-P	aminolevulinate
ALAD	aminolevulinate dehydratase
ALAS	aminolevulinate synthase
CEP	congenital erythropoetic porphyria
CPOX	coproporphyrinogen oxidase
EPP	erythropoetic porphyria
FECH	ferrochelatase
HCP	hereditary coproporphyria
HEP	hepatoerythropoetic porphyria
HMB	Hydroxymethylbilane synthase
PBG	porphobilinogen
PBGD	porphobilinogen deaminase
PCT	porphyria cutanea tarda
PPOX	protoporphyrinogen oxidase
UROD	uroporphyrinogen decarboxylase
UROS	uroporphyrinogen III synthase
VP	variegate porphyria
XLPP	X-linked protoporphyria
XLSA	X-linked sideroblastic anemia